Full Paper

Conformationally Constrained Analogs of *N*-Substituted Piperazinylquinolones: Synthesis and Antibacterial Activity of *N*-(2,3-Dihydro-4-hydroxyimino-4*H*-1-benzopyran-3-yl)-piperazinylquinolones

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A series of novel quinolone agents bearing a particular bulky and conformationally constrained bicyclic substituent (2,3-dihydro-4-hydroxyimino-4*H*-1-benzopyran-3-yl- moiety) on the piperazine ring of 7-piperazinyl quinolones (norfloxacin, enoxacin, ciprofloxacin, and levofloxacin) were synthesized and evaluated against a panel of Gram-positive and Gram-negative bacteria. Among these derivatives, ciprofloxacin counterpart **9c**, highly inhibited the tested Gram-positive bacteria, superior to that of the reference drugs, and displayed antibacterial activity at non-cytotoxic concentrations.

Keywords: Antibacterial activity / Conformationally constrained analogs / 2,3-Dihydro-4*H*-1-benzopyran / Quinolones

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Introduction

Because of increasing prevalence of bacterial infections, particularly those such as the clinically important pathogenic bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA), drug-discovery efforts have been intensified in the past years to search for more effective antibacterial agents with a broad spectrum of activity, and activity against resistant pathogens [1, 2]. The ideal strategy to such challenges is to find novel agents that inhibit new targets in bacteria. However, despite advances in drug development, finding new antibacterial agents with novel mechanisms of action remains extremely difficult. A more practical approach to such challenges is modification of the structure of existing antibacterial agents to increase potency, and to overcome resistance. Fluoroquinolones are among the most attractive agents in the treatment of bacterial infections since the discovery of norfloxacin **1** and enoxacin **2**; they appear to be an ideal subject for such explorations [3].

The bactericidal activity generated by fluoroquinolones is caused by the inhibition of two bacterial enzymes: DNA gyrase and topoisomerase IV. They inhibit the enzyme function by binding to the intermediate cata-

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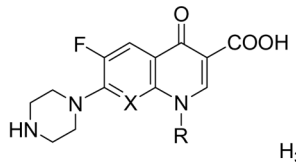
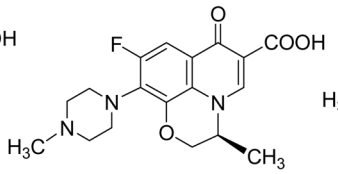
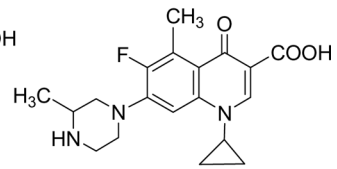
Abbreviations: methicillin-resistant *Staphylococcus aureus* (MRSA); minimum inhibitory concentration (MIC)



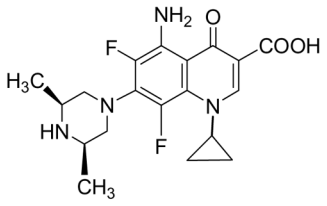
1, Norfloxacin; R= Et, X= CH
2, Enoxacin; R= Et, X= N
3, Ciprofloxacin; R= *c*-Pr, X= CH



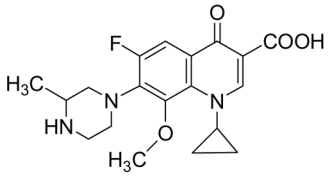
4, Levofloxacin



5, Grepafloxacin

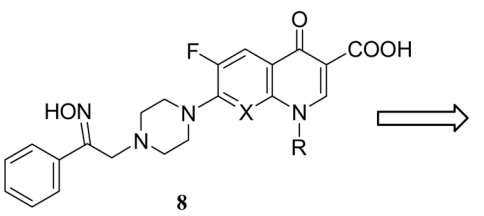


6, Sparfloxacin



7, Gatifloxacin

Figure 1. Structure of some piperazinyl quinolone antibacterials.



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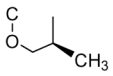
X= CH, N; R= cyclopropyl, ethyl

X & R =



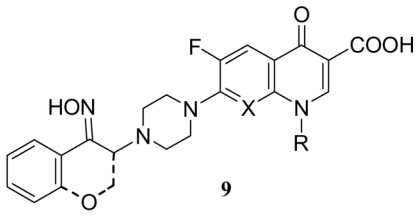


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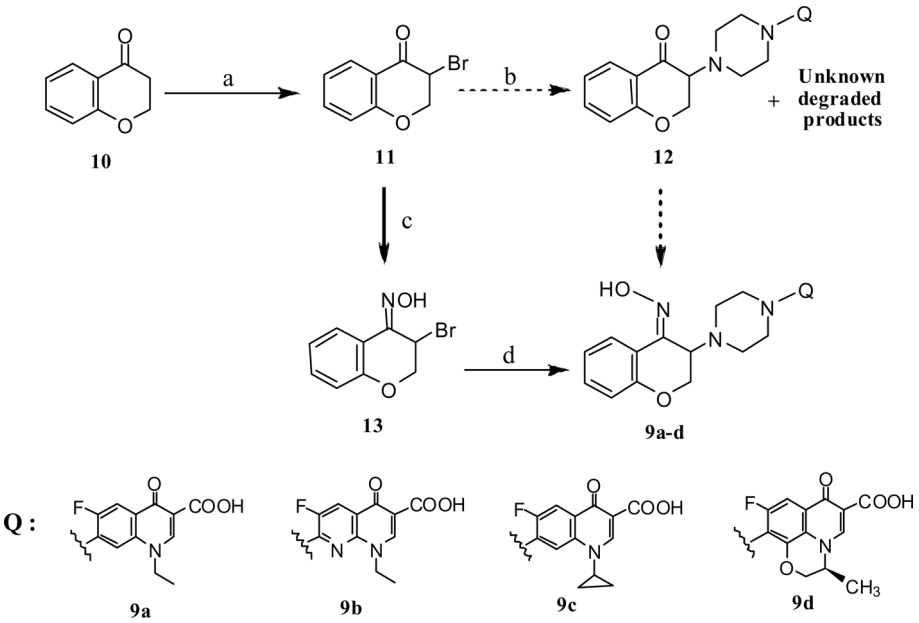
Figure 2. Design of *N*-(2,3-dihydro-4-hydroxyimino-4*H*-1-benzopyran-3-yl)-piperazinyl quinolones **9** as conformationally constrained analogs of *N*-(phenethyl)piperazinyl quinolones **8**.

lytic enzyme-DNA complex. The stabilization of the resulting quinolone-enzyme-DNA complex leads to the generation of double-strand DNA breaks that trigger a cascade of events leading to cell death [4, 5].

Although the fluoroquinolone class of antibacterials is an important weapon against bacterial infections, in particular ciprofloxacin **3** and levofloxacin **4** (Fig. 1), resistance is increasing in this class as well [6]. Studies have shown that up to 80% of MRSA in the United States are also resistant to ciprofloxacin [7, 8]. On the other hand, some more recently approved fluoroquinolones have had concerns with unacceptable side effects, for example, grepafloxacin **5** and sparfloxacin **6** were withdrawn from the market, due to increased cases of heart problems in clinical findings. Similarly, dysglycemia has been noted as the life-threatening adverse effect of gatifloxacin **7**, which led to its withdrawal from the market in the United States in 2006 [9–12].

The structure–activity relationships studies of fluoroquinolones demonstrated that substituents at the C-7 position greatly influenced their potency, spectrum, and safety [13]. One of the most important features of the fluoroquinolones is the presence of a nitrogen heterocycle, particularly a piperazinyl substituent, at the C-7 of the quinolone nucleus. The addition of a piperazinyl substituent at the C-7 position improves the ability of the quinolone to penetrate the bacterial cell wall, thereby boosting its activity against Gram-negative bacteria and providing some degree of Gram-positive activity. Thus, piperazinyl-type quinolones such as norfloxacin **1**, enoxacin **2**, ciprofloxacin **3**, and levofloxacin **4** display good Gram-negative coverage but a rather average Gram-positive spectrum [14–16].

In our ongoing antibacterial research program, we have recently developed new quinolone antibacterials **8** (Fig. 2) derived from a piperazinyl quinolone nucleus (norfloxacin **1**, enoxacin **2**, ciprofloxacin **3**, and levofloxacin **4**) bearing 2-hydroxyimino-2-phenylethyl moieties at the N-4 position of the piperazine ring and displaying *in-vitro* activity against Gram-positive organisms compara-



Reagents and conditions: a) $CuBr_2$, EtOAc/$ChCl_3$, reflux; b) norfloxaxin **1**, DMF or MeCN, $NaHCO_3$, rt; c) Hydroxylamine HCl, MeOH/H_2O, rt; d) Piperazinyl quinolones (norfloxacin **1**, enoxacin **2**, ciprofloxacin **3**, or *N*-desmethyllevofloxacin **14**), DMF, $NaHCO_3$, rt.

Scheme 1. Convenient strategy for the synthesis of *N*-(2,3-dihydro-4-hydroxyimino-4*H*-1-benzopyran-3-yl)-piperazinyl quinolones **9a – d**.

ble or higher than the respective parent quinolones [17 – 19].

Conformational restriction of bioactive molecules offers the possibility of generating structures with increased potency. These considerations prompted us to introduce restrictions in the mobility of the 2-hydroxyimino-2-phenylethyl moieties of **8** by conversion of the latter to a series of bicyclic derivatives (Fig. 2). Thus herein, we report the synthesis and antibacterial activity of *N*-(2,3-dihydro-4-hydroxyimino-4*H*-1-benzopyran-3-yl)-piperazinyl quinolones **9** as conformationally constrained analogs of *N*-(phenethyl)piperazinyl quinolones **8**.

Results and discussion

Chemistry

The synthesis of *N*-(2,3-dihydro-4-hydroxyimino-4*H*-1-benzopyran-3-yl)-piperazinyl quinolones **9** was first attempted by reaction of the corresponding 3-bromo-2,3-dihydro-4*H*-1-benzopyran-4-one **11** with piperazinyl quinolone **1** and subsequent oximation (Scheme 1). In the first step, different solvents, including DMF or MeCN were used in the presence of $NaHCO_3$, which resulted in poor yields with unknown degraded products. The alternative strategy was the introduction of an oxime functional group onto the benzopyran ring in the first step, and then, a coupling reaction with piperazinyl quinolone. Along this route, stirring a mixture of compound **11** and three equivalents of hydroxylamine hydrochloride in methanol / water at room temperature gave 3-bromo-2,3-dihydro-4*H*-1-benzopyran-4-one oxime **13**. Reaction of **13** with piperazinyl quinolones (norfloxacin **1**, enoxacin **2**, ciprofloxacin **3**, or *N*-desmethyl levofloxacin **14**) in the presence of $NaHCO_3$ in DMF at room temperature afforded *N*-(2,3-dihydro-4-hydroxyimino-4*H*-1-benzopyran-3-yl)-piperazinyl quinolones **9a – d** in good yields.

^{1}H-NMR spectroscopy is generally used to assign the (*E*)- and (*Z*)-geometry of oximes [20, 21]. Surveying similar oximes (chroman-4-one oxime systems) suggests that the proximity of the C-5 proton to the oxygen of the oxime in the α-syn configuration will deshield the proton [22, 23]. Examination of the proton at the C-5 position of the chroman ring indicated that the chemical shifts of compounds **9a – d** (δ = 8.49 – 8.51 ppm) were virtually downfield with respect to the expected chemical shift (at approx. 8.0 ppm) for the C-5 proton of the chromanone ring. Thus, the stereochemistry of the oxime moiety of compounds **9a – d** was assigned to be of *E*-orientation.

Table 1. *In-vitro* antibacterial activities of compounds **9a–d** and reference drugs against selected strains (MICs in μg/mL).

Microorganisms	9a	9b	9c	9d	1	2	3	4	14[a)]
Staphylococcus aureus	0.39	0.78	0.049	0.19	0.39	0.78	0.19	0.25	2
MRSA	0.78	0.78	0.098	0.39	0.78	1.56	0.39	Nt[#]	Nt[b)]
Staphylococcus epidermidis	0.78	0.78	0.049	0.19	0.78	0.78	0.39	0.25	1
Bacillus subtilis	0.39	0.19	0.024	0.19	0.39	0.78	0.19	0.39	0.78
Escherichia coli	3.13	12.5	0.78	3.13	0.049	0.098	0.012	0.049	0.049
Klebsiella pneumoniae	0.39	0.78	0.098	0.19	0.024	0.049	0.003	0.25	0.25
Pseudomonas aeruginosa	50	>100	50	100	3.13	6.25	0.78	3.13	>3.13

a) *N*-desmethyl levofloxacin.
b) Not tested.

Biological activity

Compounds **9a–d** were evaluated for their *in-vitro* antibacterial activity against *Staphylococcus aureus* ATCC 6538p, methicillin-resistant *Staphylococcus aureus* (MRSA, clinical isolate), *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 8739, *Klebsiella pneumoniae* ATCC 10031, and *Pseudomonas aeruginosa* ATCC 9027 using the conventional agar-dilution method [24]. The MIC (minimum inhibitory concentration) values were determined by comparison to the parent quinolones (**1–4** and *N*-desmethyl levofloxacin **14**) as reference drugs. The MICs (μg/mL) are presented in Table 1.

The MIC values of the novel *N*-(2,3-dihydro-4-hydroxyimino-4*H*-1-benzopyran-3-yl)-piperazinyl quinolones **9a–d** indicate that all target compounds showed significant antibacterial activity against Gram-positive microorganisms (including *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Bacillus subtilis*) and possess MIC values in the range of 0.024–0.78 μg/mL. Among them, the ciprofloxacin counterpart **9c** demonstrated an excellent antimicrobial activity against staphylococci (MIC = 0.049 μg/mL) and *Bacillus subtilis* (MIC = 0.024 μg/mL). It is worth noting that compounds **9a–d** are potent also towards clinical isolate of methicillin-resistant *Staphylococcus aureus* (MRSA) that are inhibited by all compounds at concentrations of 0.098–0.78 μg/mL.

Comparison between MICs of the compounds **9a–d** and corresponding parent piperazinyl quinolones (**1–3** and **14**) against Gram-positive microorganisms revealed that incorporation of the *N*-(2,3-dihydro-4-hydroxyimino-4*H*-1-benzopyran-3-yl)-moiety on to the piperazine ring of ciprofloxacin **3** and *N*-desmethyl levofloxacin **14** increases the activity 4 to 10 times. While norfloxacin and enoxacin counterparts (**9a** and **9b**) are equivalent in antibacterial activity against Gram-positive bacteria with respect to their parent quinolones (**1** and **2**, respectively).

Compounds **9a–d** showed poor activity, expressed as minimal inhibitory concentrations, against *Pseudomonas aeruginosa* (MIC $\geq$ 50 μg/mL), whereas they exhibited a respectable effectiveness towards other Gram-negative bacteria. More significant inhibitory properties were detected for the ciprofloxacin derivative **9c** against *Escherichia coli* (MIC = 0.78 μg/mL) and *Klebsiella pneumoniae* (MIC = 0.098 μg/mL). Generally, compounds **9a–d** were less active than the reference drugs against Gram-negative bacteria.

The cytotoxic activities of compounds **9a–d** were also assessed in comparison with the reference drug ciprofloxacin **3** using the MTT colorimetric assay against normal mouse fibroblasts (NIH/3T3) [25, 26]. The IC_{50} values obtained for these compounds are shown in Table 2. As can be seen from the results, the IC_{50} values of the compounds were close together and there was no significant cytotoxicity at concentrations below 120 μg/mL. The results showed compound **9d** to be slightly less toxic than the other compounds and reference drug ciprofloxacin. By comparing the results of toxicity and antibacterial assays, it is seen that these compounds display antibacterial activity at non-cytotoxic concentrations.

It is believed that drugs cross biological barriers most frequently through passive transport, which strongly depends on their physicochemical properties. The physicochemical properties of quinolones (e. g., hydrophobicity and molecular mass) are important for penetration into the bacterial cell and have a different role in Gram-negative and Gram-positive bacteria [15]. The lipophilicity describes the partitioning of a compound between an aqueous and an organic phase, and is characterized by the octanol / water partition coefficient. In this context, the balance of hydrophobicity would be important for such an activity [27]. The logP of the target compound, that is the logarithm of the partition coefficient for *n*-octanol / water, was calculated using the program ChemDraw Ultra 8.0 and the obtained results are given in Table 2. The logP values for the ciprofloxacin derivative **9c** was about 1.47 times higher than for the corresponding parent quinolone **3**. It could be assumed that the

Table 2. *In vitro* cytotoxic activity of compounds **9a** – **d** and ciprofloxacin **3** against mouse fibroblast (NIH/3T3) cell line.

Compound	IC_{50} (μg/mL)[a)]	LogP[b)]
9a	145 ± 19	2.83
9b	120 ± 20	2.93
9c	146 ± 6	2.79
9d	175 ± 5	2.44
3	164 ± 3.5	1.32

a) IC_{50} is the concentration required to inhibit 50% of cell growth. The values represent mean ± standard deviation of triplicate determinations.

b) LogP, that is the logarithm of the partition coefficient for *n*-octanol / water, was calculated using the program ChemDraw Ultra 8.0.

increase in bulkiness of the substituent at the C-7 position and the lipophilicity (logP) of the compound **9c** with respect to the parent quinolone **3**, results in the increase of antibacterial activity against Gram-positives bacteria. These do not possess an outer membrane i. e., they lack outer membrane proteins and lipopolysaccharides. Accumulation by Gram-positive bacteria (e. g., *S. aureus*) is thought to take place by simple diffusion across the cytoplasmic membrane. Accordingly, we suggest that compounds like **9c** containing bulky substituent at C-7 position, which have high hydrophobicity and molecular mass, are accumulated in the Gram-positive bacteria more favorably than ciprofloxacin.

Conclusions

In light of this study, it can be inferred that the antibacterial properties of target compounds are determined by a combination of influences: the N-1 substituent (ethyl or cyclopropyl), position 8 of quinolone nucleus, and a conformationally constrained substituent (2,3-dihydro-4-hydroxyimino-4*H*-1-benzopyran-3-yl) on the piperazine ring. Thus, the *N*-(2,3-dihydro-4-hydroxyimino-4*H*-1-benzopyran-3-yl)-pendent group is well tolerated at the N-4 position of the piperazine ring in some 7-piperazinyl quinolones (exemplified by ciprofloxacin and *N*-desmethyl levofloxacin), and enhances antibacterial potency against Gram-positive bacteria. Indeed, we identified that incorporation of a *N*-(2,3-dihydro-4-hydroxyimino-4*H*-1-benzopyran-3-yl)-scaffold as bulky and conformationally constrained bicyclic substituent on the piperazine ring allows manipulation of selectivity and potency of 7-piperazinyl quinolones.

In summary, we have synthesized novel quinolone agents bearing a particular bulky and conformationally constrained bicyclic substituent (2,3-dihydro-4-hydroxyimino-4*H*-1-benzopyran-3-yl-moiety) on the piperazine ring of the 7-piperazinyl quinolones. *In-vitro* antibacterial and cytotoxic evaluation of target compounds showed that this scaffold served as a promising C-7 substituent for piperazinyl quinolones. Among these derivatives, the ciprofloxacin counterpart **9c**, showed a high inhibition of all the tested Gram-positive bacteria, superior to the reference drugs, and displayed antibacterial activity at non-cytotoxic concentrations.

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Experimental

Chemistry

All chemical reagents and solvents used in this study were purchased from Merck AG (Darmstadt, Germany). The starting materials **1** – **3** and **10** were purchased from Aldrich Chemical Company. The *N*-desmethyl levofloxacin **14** was prepared according to the literature method [28]. Melting points were determined in open glass capillaries using Bibby Stuart Scientific SMP3 apparatus (Bibby Sterlin Ltd. UK) and are uncorrected. The IR spectra were obtained on a Shimadzu 470 spectrophotometer (potassium bromide disks) (Shimadzu, Tokyo, Japan). NMR spectra were appropriately recorded using a Bruker 80 or Bruker 500 spectrometer (Bruker Bioscience, Billerica, MA, USA), and chemical shifts are expressed as δ (ppm) with tetramethylsilane as internal standard. Elemental analyses were carried out on a HERAEUS CHN-O rapid elemental analyzer (Heraeus GmbH, Hanau, Germany) for C, H and N, and the results are within ± 0.4% of the theoretical values. Merck silica gel 60 F254 plates were used for analytical TLC.

*3-Bromo-2,3-dihydro-4H-1-benzopyran-4-one **11***

A vigorously stirred mixture of pulverized copper (II) bromide (22.34 g, 100 mmol) and chroman-4-one **10** (8.88 g, 60 mmol) in chloroform / ethyl acetate (1 : 1, 100 mL) was refluxed until the reaction was completed as judged by a color change of the solution from green to amber, disappearance of all black solid, and cessation of HBr evolution (2 h). After removal of the copper (I) bromide (white solid) by filtration, the solvents were evaporated from the filtrate under reduced pressure. The residue was crystallized from *n*-heptane / diethyl ether to give compound **11**.

Yield: 70%; m. p.: 68 – 69°C; ^{1}H-NMR (80 MHz, $CDCl_3$) δ: 4.40 – 4.80 (m, 3H, OCH_2CH), 6.90 – 7.18 (m, 2H, H-6 and H-8), 7.54 (dt, *J* = 8.0, 1.8 Hz, 1H, H-7), 7.95 (dd, *J* = 8.0, 1.8 Hz, 1H, H-5).

*3-Bromo-2,3-dihydro-4H-1-benzopyran-4-one oxime **13***

A solution of **11** (0.45 g, 2.0 mmol) and hydroxylamine hydrochloride (0.42 g, 6.0 mmol) in methanol (6 mL) was stirred at room temperature for 48 h and then water (2 mL) was added and stirring was continued for additional 24 h. After consumption of **11** (monitored by TLC), water (10 mL) was added and the precipitate was filtered, washed with water and dried to give **13**. Yield: 90%; m. p.: 168 – 170°C; ^{1}H-NMR (80 MHz, $CDCl_3$) δ: 4.30 (dd, *J* =

12.8, 1.8 Hz, 1H, H-2a), 4.52 (dd, *J* = 12.8, 1.8 Hz, 1H, H-2b), 5.48 (t, *J* = 1.8 Hz, 1H, H-3), 6.80–7.10 (m, 2H, H-6 and H-8), 7.31 (dt, *J* = 6.4, 2.4 Hz, 1H, H-7), 7.83 (dd, *J* = 8.4, 2.4 Hz, 1H, H-5).

General procedure for the synthesis of N-(2,3-dihydro-4-hydroxyimino-4H-1-benzopyran-3-yl)-piperazinylquinolones ***9a–d***

A mixture of 3-bromo-2,3-dihydro-*4H*-1-benzopyran-4-one oxime **13** (0.5 mmol), piperazinylquinolone (**1–3** or *N*-desmethyl levofloxacin **14**, 0.5 mmol) and $NaHCO_3$ (0.5 mmol) in DMF (5 mL), was stirred at room temperature for 24–72 h. After consumption of piperazinylquinolone (monitored by TLC), water (10 mL) was added and the precipitate was filtered, washed with water and crystallized from DMF / water to give compound **9a–d** as (*E*)-oximes.

1,4-Dihydro-1-ethyl-6-fluoro-7-[4-(4-hydroxyimino-2,3-dihydro-4H-1-benzopyran-3-yl)piperazin-1-yl]-4-oxo-3-quinoline carboxylic acid ***9a***

(*E*)-Isomer; yield: 93%; m.p.: 238–239°C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 1.38 (t, *J* = 6.97 Hz, 3H, -CH_3 ethyl), 2.65–2.83 (m, 4H, piperazine), 3.06 (s, 1H, H-3 chroman), 3.20–3.28 (m, 4H, piperazine), 4.16 (d, *J* = 12.20 Hz, 1H, H-2a chroman), 4.54 (q, *J* = 6.86 Hz, 2H, -CH_2-ethyl), 4.84 (d, *J* = 12.27 Hz, 1H, H-2b chroman), 6.90 (d, *J* = 8.26 Hz, 1H, H-8 chroman), 6.95 (t, *J* = 7.57 Hz, 1H, H-6 chroman), 7.15 (d, *J* = 6.94 Hz, 1H, H-8 quinoline), 7.30 (t, *J* = 7.71 Hz, 1H, H-7 chroman), 7.90 (d, *J* = 13.10 Hz, 1H, H-5 quinoline), 8.49 (d, *J* = 7.89 Hz, 1H, H-5 chroman), 8.93 (s, 1H, H-2 quinoline), 11.54 (s, 1H, OH oxime), 15.31 (s, 1H, COOH). Anal. calcd. for $C_{25}H_{25}FN_4O_5$: C, 62.49; H, 5.24; N, 11.66. Found: C, 62.63; H, 5.11; N, 11.83.

1,4-Dihydro-1-ethyl-6-fluoro-7-[4-(4-hydroxyimino-2,3-dihydro-4H-1-benzopyran-3-yl)piperazin-1-yl]-4-oxo-1,8-naphthyridine-3-carboxylic acid ***9b***

(*E*)-Isomer; yield: 96%; m.p.: 206–207°C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 1.36 (t, *J* = 7.00 Hz, 3H, -CH_3 ethyl), 2.60–2.82 (m, 4H, piperazine), 3.04 (s, 1H, H-3 chroman), 3.69–3.82 (m, 4H, piperazine), 4.15 (d, *J* = 11.94 Hz, 1H, H-2a chroman), 4.46 (q, *J* = 6.99 Hz, 2H, -CH_2- ethyl), 4.82 (d, *J* = 12.29 Hz, 1H, H-2b chroman), 6.91 (d, *J* = 8.26 Hz, 1H, H-8 chroman), 6.95 (t, *J* = 7.65 Hz, 1H, H-6 chroman), 7.31 (t, *J* = 7.24 Hz, 1H, H-7 chroman), 8.05 (d, *J* = 13.49 Hz, 1H, H-5 naphthyridine), 8.50 (d, *J* = 8.00 Hz, 1H, H-5 chroman), 8.95 (s, 1H, H-2 naphthyridine), 11.56 (s, 1H, OH oxime), 15.30 (s, 1H, COOH). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 15.53, 47.34, 47.40, 48.06, 50.79, 61.82, 67.37, 108.90, 113.43, 115.83, 117.40, 120.16, 120.33, 120.89, 131.88, 131.93, 144.91, 145.68, 147.05 148.55, 150.03, 155.918, 166.72, 177.17. Anal. calcd. for $C_{24}H_{24}FN_5O_5$: C, 59.87; H, 5.02; N, 14.55. Found: C, 60.01; H, 4.89; N, 14.50.

1-Cyclopropyl-1,4-dihydro-6-fluoro-7-[4-(4-hydroxyimino-2,3-dihydro-4H-1-benzopyran-3-yl)piperazin-1-yl]-4-oxo-3-quinoline carboxylic acid ***9c***

(*E*)-Isomer; yield: 61%; m.p.: 245–246°C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 1.10–1.20 (m, 2H, cyclopropyl), 1.26–1.32 (m, 2H, cyclopropyl), 2.66–2.86 (m, 4H, piperazine), 3.07 (s, 1H, H-3 chroman), 3.20–3.28 (m, 4H, piperazine), 3.76 (br s, 1H, cyclopropyl), 4.16 (d, *J* = 12.10 Hz, 1H, H-2a chroman), 4.84 (d, *J* = 12.10 Hz, 1H, H-2b chroman), 6.91 (d, *J* = 8.24 Hz, 1H, H-8 chroman), 6.95 (t, *J* = 7.62 Hz, 1H, H-6 chroman), 7.31 (t, *J* = 7.25 Hz, 1H, H-7 chroman), 7.54 (d, *J* = 7.17 Hz, 1H, H-8 quinoline), 7.89 (d, *J* = 13.13 Hz, 1H, H-5 quinoline), 8.51 (d, *J* = 7.35 Hz, 1H, H-5 chroman), 8.65 (s, 1H, H-2 quinoline), 11.56 (s, 1H, OH oxime), 15.20 (s, 1H, COOH). Anal. calcd. for $C_{26}H_{25}FN_4O_5$: C, 63.41; H, 5.12; N, 11.38. Found: C, 63.35; H, 5.19; N, 11.22.

2,3-Dihydro-9-fluoro-3-methyl-10-[4-(4-hydroxyimino-2,3-dihydro-4H-1-benzopyran-3-yl)piperazin-1-yl]-7-oxo-7H-pyrido[1,2,3-de][1,4]benzoxazine-6-carboxylic acid ***9d***

(*E*)-Isomer; yield: 95%; m.p.: 216–217°C; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 1.43 (d, *J* = 5.98 Hz, 3H, CH_3), 2.55–2.81 (m, 4H, piperazine), 3.05 (s, 1H, H-3 chroman), 3.20–3.29 (m, 4H, piperazine), 4.15 (d, *J* = 12.25 Hz, 1H, H-2a chroman), 4.34 (d, *J* = 9.68 Hz, 1H, H-2a pyridobenzoxazine), 4.55 (d, *J* = 10.57 Hz, 1H, H-2b pyridobenzoxazine), 4.81 (d, *J* = 11.0 Hz, 1H, H-2b chroman), 4.82–4.99 (m, 1H, H-3 benzoxazine), 6.91 (d, *J* = 8.22 Hz, 1H, H-8 chroman), 6.95 (t, *J* = 7.55 Hz, 1H, H-6 chroman), 7.31 (t, *J* = 7.63 Hz, 1H, H-7 chroman), 7.55 (d, *J* = 12.07 Hz, 1H, H-8 pyridobenzoxazine), 8.50 (d, *J* = 7.97 Hz, 1H, H-5 chroman), 8.94 (s, 1H, H-5 pyridobenzoxazine), 11.53 (s, 1H, OH oxime), 15.17 (s, 1H, COOH). ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 18.74, 51.02, 51.63, 55.67, 62.05, 67.54, 68.91, 104.21, 115.92, 117.34, 120.83, 125.61, 131.84, 131.88, 141.09, 145.08, 147.05, 155.96, 166.92, 177.22. Anal. calcd. for $C_{26}H_{25}FN_4O_6$: C, 61.41; H, 4.96; N, 11.02. Found: C, 61.57; H, 5.11; N, 10.88.

Antibacterial activity

Compounds **9a–d** were evaluated for their *in-vitro* antibacterial activity against selected strains using conventional agar-dilution method in comparison to the reference drugs [24]. Drugs (10.0 mg) were dissolved in DMSO (1 mL) and the solution was diluted with water (9 mL). Further progressive twofold serial dilution with melted Mueller–Hinton agar was performed to obtain the required concentrations of 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.19, 0.098, 0.049, 0.024, 0.012, 0.006, 0.003, and 0.0015 μg/mL. The bacteria inocula were prepared by suspending overnight colonies from Mueller–Hinton agar media in 0.85% saline. The inocula were adjusted photometrically at 600 nm to a cell-density equivalent of approximately 0.5 McFarland standard (1.5×10^8 CFU/mL). The suspensions were then diluted in 0.85% saline to give 10^7 CFU/mL. Petri dishes were spot-inoculated with 1 μL of each prepared bacterial suspension (10^4 CFU/spot) and incubated at 35–37°C for 18 h. The minimum inhibitory concentration (MIC) was the lowest concentration of the test compound, which resulted in no visible growth on the plate. To ensure that the solvent had no effect on bacterial growth, a control test was performed with test medium supplemented with DMSO at the same dilutions as used in the experiment.

MTT colorimetric assay

The cytotoxic activity of target compounds were assessed using MTT colorimetric assay [25] against normal mouse fibroblasts (NIH/3T3). Briefly, cultures in the exponential growth phase were trypsinized and diluted in complete growth medium to give a total cell count of 5×10^4 cells/mL. 100 μL of suspension was added to the wells of sterile 96-well plates. After plating, 50 μL of a serial dilution of every agent was added. Each compound dilution was assessed in triplicate. Three wells containing only normal mouse fibroblast (NIH/3T3) cells suspended in

150 μL of complete medium were used as control for cell viability. The plates were then incubated for 72 h. After incubation, 30 μL of a 5 mg/mL solution of MTT was added to each well and the plate was incubated for another 1 h. After incubation, the culture medium was replaced with 100 μL of DMSO. Then, the absorbance of each well was measured by using a micro-plate reader at 492 nm wavelengths. For each compound, a dose-response curve was measured with different drug concentrations, and the concentration causing 50% cell growth inhibition (IC_{50}) compared with the control was calculated.

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