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Original article

Synthesis, antibacterial, antifungal and anti-HIV activities of norfloxacin Mannich bases

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Abstract – Mannich bases of norfloxacin were synthesized by reacting them with formaldehyde and several isatin derivatives. Their chemical structures have been confirmed by means of their IR, ^{1}H-NMR data and by elemental analysis. Investigation of in vitro antimicrobial activity of compounds was done by the agar dilution method against 28 pathogenic bacteria, eight pathogenic fungi and anti-HIV activity against replication of HIV-1 (III B) in MT-4 cells. The in vivo antibacterial efficacy of selected derivatives was determined using a mouse infection model. All the synthesized compounds are more active than norfloxacin against the 13 bacteria tested. The compounds are also more active than the standard drug clotrimazole against *Histoplasma capsulatum*. Two compounds **S-8** and **S-9** have shown inhibition against HIV-1 (III B) with EC_{50} values of 11.3 and 13.9 μg/mL, respectively. In the mouse protection test, two compounds **S-4** (ED_{50}: 1.25 mg/kg) and **S-9** (ED_{50}: 1.62 mg/kg) are more active than norfloxacin (ED_{50}: 6mg/kg). Among the compounds tested, 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7[[N^4-[5'-bromo-3'-(4'-amino-5'-trimethoxybenzylpyrimidin-2'-yl]-imino-1'-isatinyl]methyl]N^1-piperazinyl]-3-quinoline carboxylicacid (**S-9**) showed promising activity in all the three tests. © 2000 Éditions scientifiques et médicales Elsevier SAS

Mannich bases / norfloxacin / isatin / antimicrobial

1. Introduction

The fluoroquinolone antibacterial agents have been found to be one of the fastest growing group of drugs in recent years. To date, more than 10 000 different analogues have been synthesized. They are unusual in being totally synthesized chemically. This means that various side chains can be altered and the resulting analogues tested for their antimicrobial properties. Most of these agents are substituted at the 7 position by a nitrogen heterocycle. Norfloxacin [1], characterized by having a piperazine moiety at C-7, which represents a site amenable to significant modification. We report our results from a study of replacing the N^4-hydrogen of piperazine with various isatin derivatives. Isatin derivatives are reported to show antibacterial [2, 3], antifungal [4], and anti-HIV activities [5–7]. Fluoroquinolone derivatives also exhibit inhibitory activity against human immunodeficiency virus type 1 [8]. In the present study we have aimed to achieve a better antimicrobial profile at lower concentrations, by preparing Mannich bases with isatin derivatives. This report deals with the synthesis of Mannich bases of norfloxacin and screening for their antibacterial, antifungal and anti-HIV activities.

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2. Chemistry

The starting materials 3-[4-sulfadiazinimino] isatin, 3-[4(-sulfadoximino] isatin and 3-[4'-amino,5'-(3", 4",5"-trimethoxybenzyl)pyrimidin-2'-yl]imino isatin and their 5 substituted derivatives were prepared according to the literature methods [9–11] by reacting isatin and its derivatives with sulfadiazine, sulfadoxine and trimethoprim in the presence of glacial acetic acid. Mannich bases of norfloxacin were prepared by condensing the active hydrogen atom of isatin derivatives with formaldhyde

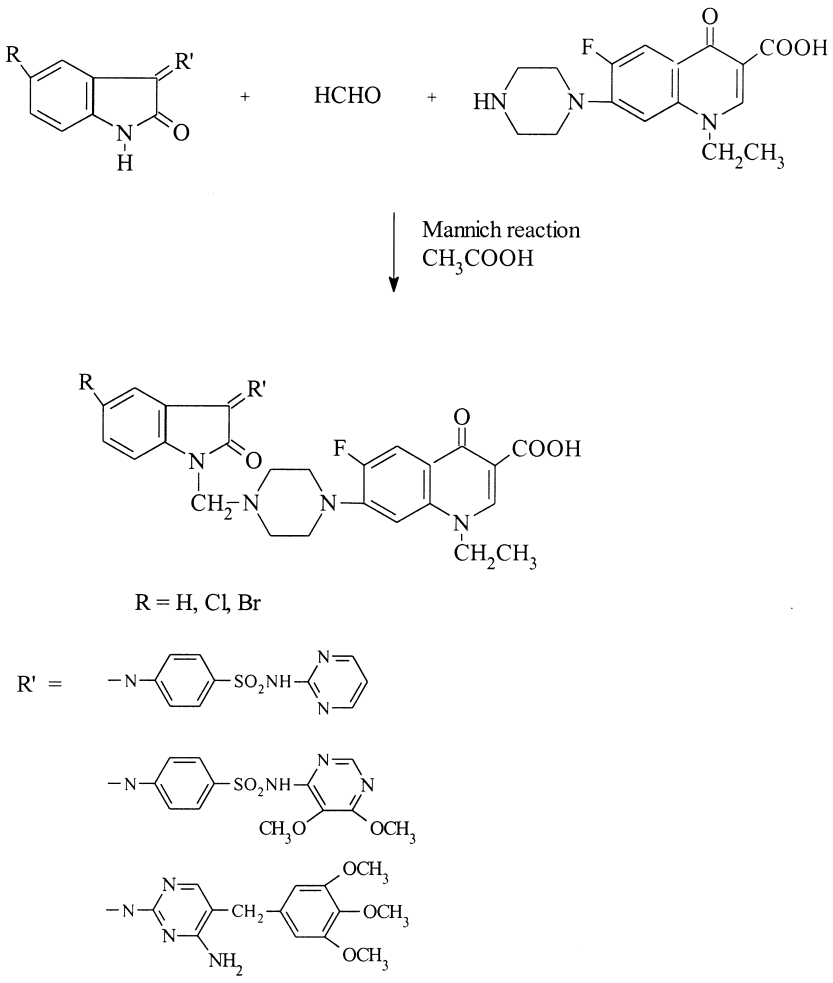


Figure 1. Protocol for the synthetic compounds.

and the secondary amino function (piperazino moiety) of norfloxacin (*figure 1*).

3. Biological investigation and discussion

Compounds **S1–S9** were evaluated for their in vitro antibacterial activity against 28 pathogenic bacteria by conventional agar dilution procedures and the results of these assays are summerized in *table I*. The data for norfloxacin are included for comparison. The observation from the antibacterial activity of norfloxacin Mannich bases showed very good activity against tested bacteria. All the compounds are more active than norfloxacin against *Vibrio parahaemolyticus, Edwardsiella tarda, Vibrio cholerae* 0139, *Staphylococcus aureus, Escherichia coli* NCTC 10418, *Streptococcus faecalis, Aeromonas hydrophila, Shigella sonnei, Plesiomonas shigelloides, Proteus rettgeri, Shigella flexneri, Morgonella morganii* and *Salmonella paratyphi* A. All the compounds are more active than norfloxacin (MIC: 9.76 μg/mL) against *E. tarda* and *P. rettgeri* (0.075–1.22 μg/mL), *V. cholerae* 0139 (0.018–1.22 μg/mL), *S. faecalis* (0.15–2.44 μg/mL), *S. sonnei* (0.61–4.88 μg/mL), *P. shigelloides* (0.037–0.3 μg/mL) and *S. paratyphi* A (0.037–2.44 μg/mL). The synthesized compounds are more active than norfloxacin (2.44 μg/mL) against *V. parahaemolyticus* (0.3–1.22 μg/mL), *Shigella flexneri* (0.018–1.22 μg/mL) and *Morgonella morganii* (0.018–0.6 μg/mL). The compounds are also more active than norfloxacin (0.3 μg/mL) against *Escherichia coli* (≤ 0.018–0.15 μg/mL) and *A. hydrophile* (≤ 0.018–0.075 μg/mL). Seven compounds (**S3–S9**) are more active (312.5–

Table I. Biological testing results from antibacterial screening: MICs in μg/mL.

Micro-organism	Drugs									
	S-1	**S-2**	**S-3**	**S-4**	**S-5**	**S-6**	**S-7**	**S-8**	**S-9**	Nor-floxacin
Salmonella typhimurium	0.3	0.3	0.3	2.44	2.44	1.22	0.61	0.61	0.15	0.3
Vibrio parahaemolyticus	0.3	0.61	0.3	1.22	1.22	0.3	0.61	1.22	0.3	2.44
Salmonella paratyphi B	0.037	0.075	0.037	0.075	0.075	0.018	0.3	0.15	0.075	≤ 0.018
Edwardsiella tarda	0.15	0.15	0.075	1.22	0.30	0.61	0.6	0.3	0.075	9.76
Vibrio cholerae 0139	0.037	0.075	0.037	1.22	0.61	0.61	0.037	0.037	0.018	9.76
Staphylococcus aureus	1 250	625	625	1 250	1 250	1 250	1 250	625	625	2 500
Escherichia coli NCTC 10418	0.15	0.15	0.075	0.018	0.018	≤ 0.018	0.018	0.037	≤ 0.018	0.3
Vibrio cholerae non-01	≤ 0.018	≤ 0.018	≤ 0.018	0.037	0.018	0.018	≤ 0.018	0.018	≤ 0.018	≤ 0.018
Streptococcus faecalis	0.3	0.15	0.075	2.44	1.22	0.3	0.61	0.3	0.15	9.76
Salmonella typhi	0.018	0.018	≤ 0.018	0.61	0.61	0.61	0.037	0.037	≤ 0.018	≤ 0.018
Pseudomonas aeruginosa	39.06	19.53	19.53	78.12	78.12	39.06	78.12	78.12	78.12	19.53
Klebsiella pneumoniae	2 500	1 250	625	625	625	625	625	312.5	625	1 250
Staphylococcus albus	625	312.5	312.5	2 500	2 500	2 500	1 250	625	625	312.5
Salmonella enteritidis	0.037	0.018	≤ 0.018	0.037	0.037	0.037	0.075	0.075	0.075	≤ 0.018
Aeromonas hydrophila	≤ 0.018	≤ 0.018	≤ 0.018	0.037	≤ 0.018	≤ 0.018	0.037	0.075	0.018	0.3
Vibrio cholerae-01	0.037	0.018	≤ 0.018	≤ 0.018	≤ 0.018	≤ 0.018	0.037	0.075	0.018	≤ 0.018
Bacillus subtilis	1.22	1.22	0.61	0.15	0.15	0.3	0.15	0.075	0.018	1.22
Shigella sonnei	4.88	4.88	1.22	4.88	4.88	2.44	1.22	0.61	0.61	9.76
Shigella boydii	0.075	0.15	0.075	0.15	0.075	0.018	0.037	0.075	0.15	≤ 0.018
Plesiomonas shigelloides	0.3	0.3	0.15	0.15	0.075	0.037	0.037	0.037	0.075	9.76
Proteus rettgeri	1.22	0.61	0.61	1.22	0.6	0.075	0.6	0.15	0.075	9.76
Shigella flexneri	0.61	0.61	0.61	1.22	0.6	0.3	0.037	0.018	0.018	2.44
Proteus vulgaris	0.037	0.037	0.018	0.075	0.075	0.075	0.037	0.037	≤ 0.018	≤ 0.018
Enterobacter	0.075	0.075	0.018	0.075	0.037	0.075	0.075	0.037	≤ 0.018	≤ 0.018
Morgonella morganii	0.075	0.075	0.018	0.075	0.075	0.037	0.6	0.15	0.075	2.44
Citrobacter freundii	0.075	0.037	0.037	0.3	0.15	0.075	0.075	0.15	0.075	≤ 0.018
Proteus morgonii	0.075	0.075	0.075	0.3	0.15	0.075	0.15	0.15	0.037	0.018
Salmonella paratyphi A	0.075	0.075	0.037	0.15	0.15	0.15	2.44	0.61	0.15	9.76

MIC – Minimum inhibitory concentration

1250 μg/mL) than norfloxacin (2 500 μg/mL) against *Klebsiella pneumoniae*. Seven compounds (**S3–S9**) are more active (0.018–0.61 μg/mL) and two compounds (**S1** and **S2**) are equipotent to that of norfloxacin (1.22 μg/mL) against *Bacillus subtilis*. In general, the order of antibacterial activity of the substituents at the 5th position of isatin is Br > Cl > H. In the case of the substitution of the 3-keto derivative, order of activity is trimethoprim > sulfadiazine > sulfadoxine. Compound **S-9** showed excellent in vitro antibacterial activity. Sulfonamides and trimethoprim showed antibacterial activity by inhibiting the folate reductase, whereas quinolones exert their antibacterial activity by inhibiting the subunit A of DNA gyrase [12]. The better antibacterial activity of the synthesized Mannich bases might be due to the inhibitory effect of both the folate reductase and DNA gyrase enzymes. In vivo antibacterial activities of the Mannich bases against an experimentally induced infection of mice after oral administration are given in *table II*, together with the in vitro activity against the infecting strains. Norfloxacin was used as the reference compound. Compounds **S-4** and **S-9** are more active than norfloxacin (ED_{50}: 6 mg/kg body weight) with ED_{50}'s of 1.25 and 1.62 mg/kg body weight, respectively. From the comparison of the activities of these compounds, **S-4** is 4.8 times

Table II. In vitro and in vivo antibacterial activities and acute oral toxicities.

Compound	*E. coli* NCTC 10418 MIC, μg/mL	ED_{50}, mg/kg, p.o.	$LD_{50\ mg}$/kg	T.I.[a]
S-3	0.075	10.0	> 4 000	> 400
S-4	0.018	1.25	> 4 000	> 3 200
S-9	0.018	1.62	3 200	1 975
Norfloxacin	0.3	6.0	4 000	666

[a]Ratio of LD_{50} to ED_{50}

Table III. Antifungal activity of the compounds: MIC in μg/mL.

Drug	Micro-organism							
	Cryptococcus neoformans	*Microsporum audouinii*	*Trichophyton mentagrophytes*	*Epidermophyton floccosum*	*Microsporum gypsum*	*Histoplasma capsulatum*	*Candida albicans*	*Aspergillus niger*
S-1	2.44	9.76	156.25	4.88	9.76	4.88	156.25	156.25
S-2	2.44	19.53	156.25	4.88	4.88	2.44	156.25	156.25
S-3	2.44	9.76	156.25	2.44	2.44	2.44	156.25	78.12
S-4	1.22	0.6	39.06	2.44	1.22	1.22	156.25	9.76
S-5	2.44	0.6	19.53	4.88	1.22	2.44	156.25	39.06
S-6	1.22	0.6	39.06	2.44	0.6	1.22	312.5	19.53
S-7	2.44	0.6	19.53	4.88	2.44	4.88	156.25	78.12
S-8	0.6	0.6	9.76	1.22	9.76	1.22	156.25	78.12
S-9	0.6	0.6	4.88	1.22	2.44	2.44	78.12	19.53
Norfloxacin	4.88	19.53	156.25	19.53	19.53	19.53	312.5	156.25
Clotrimazole	2.44	4.88	2.44	2.44	2.44	19.53	0.3	2.44

MIC – Minimum inhibitory concentration

and **S-9** is 3.7 times more active than norfloxacin. The in vivo activity may be ascribed due to the increased water solubility of the corresponding Mannich bases of norfloxacin.

The results from the antifungal study (*table III*) show that all the compounds exhibited significant antifungal activity. All the Mannich bases are more potent than norfloxacin against the fungi tested. When compared to clotrimazole (MIC: 19.53 μg/mL) all the compounds are more active (1.22–2.44 μg/mL) against *Histoplasma capsulatum*. Six compounds (**S4–S9**) are more active (0.6 μg/mL) than clotrimazole (4.88 μg/mL) against *Microsporum audouinii*. When compared to clotrimazole (2.44 μg/mL), four compounds (**S4**, **S6**, **S8** and **S9**) are more active (0.6–1.22 μg/mL), five compounds (**S1–S3**, **S5** and **S7**) are equipotent against *Cryptococcus neoformans*, three compounds (**S4–S6**) are more active (0.6–1.22 μg/mL) against *Microsporum gypsum*. Compound **S-9** showed better antifungal activity when compared to other Mannich bases.

The compounds were evaluated for their inhibitory effect on the replication of HIV-1 in human MT-4 cells (*table IV*). Compounds **S-8** and **S-9** showed inhibition with an EC_{50} of 11.3 and 13.9 μg/mL, respectively, and the selectivity index is up to 5. Other compounds showed marked anti-HIV activity at a concentration below their toxicity threshold.

The acute toxicities of the selected compounds are summarized in *table II*. It is apparent that the toxicities of **S-3** and **S-4** were significantly lower than that of norfloxacin.

Table IV. Anti-HIV activity of the compounds.

Compound	EC_{50}[a], μg/mL	CC_{50}[b], μg/mL	SI[c]	Max. Prot.[d]
S-1	> 48	48.5	< 1	0
S-2	> 63	63.1	< 1	1
S-3	> 62	61.8	< 1	0
S-4	> 88	88.5	< 1	4
S-5	> 51	51.2	< 1	3
S-6	> 34	33.9	< 1	2
S-7	> 66	66.5	< 1	7
S-8	11.3	57.6	5	95
S-9	13.9	54.7	4	73

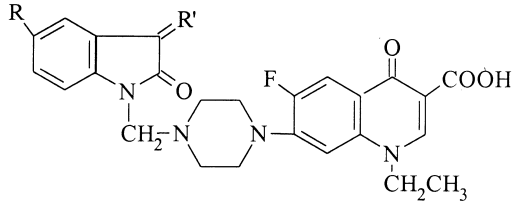
[a] Effective dose of compound achieving 50% protection in MT-4 cells against the cytopathic effect of HIV-1. [b]Cytotoxic dose of compound required to reduce the viability of mock infected MT-4 cells by 50%. [c]Selectivity index or ratio of CC_{50} to EC_{50}. [d]Maximum protection in %.

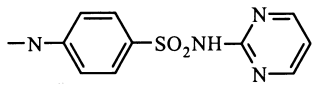
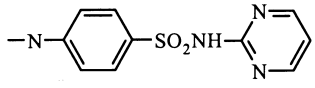
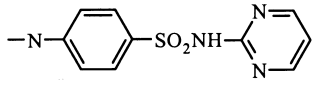
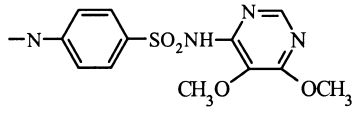
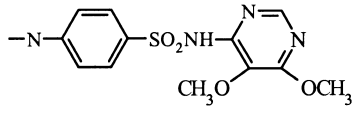
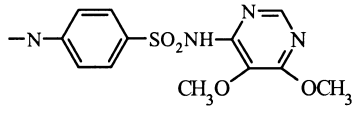
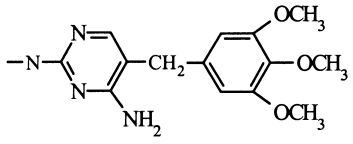
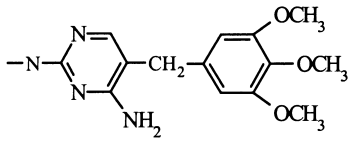
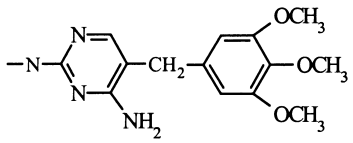
4. Experimental protocols

4.1. Chemistry

Melting points were determined in open capillary tubes on a Thomas-Hoover melting point apparatus and are uncorrected. IR spectra were recorded for KBr pellets on a Jasco IR report-100 infrared spectrophotometer. ^{1}H-NMR spectra were determined at 90 MHz on a Jeol 90Q FT spectrometer with tetramethyl silane as an internal standard. The purity of the compounds was checked by TLC on silica-gel coated glass plates visualized by iodine vapour. Microanalyses were performed by the microanalytical unit, Central Drug Research Institute, India. All compounds (*table V*) gave satisfactory elemental analyses. IR and ^{1}H-NMR spectra were consistent with the assigned structures.

Table V. Physical constants of the compounds.



No.	R	R'	Yield (%)	M.p. (°C)	Mol. formula	Rf: C_6H_6:C_2H_5OH; 9:1
S-1	H		76.56	125	$C_{35}H_{31}O_6N_8F$	0.5950
S-2	Cl		83.77	120	$C_{35}H_{30}O_6N_8FCl$	0.6097
S-3	Br		89.41	121	$C_{35}H_{30}O_6N_8FBr$	0.6226
S-4	H		67.07	119	$C_{37}H_{35}O_8N_8F$	0.6078
S-5	Cl		71.23	127	$C_{37}H_{34}O_8N_8FCl$	0.6126
S-6	Br		66.44	135	$C_{37}H_{34}O_8N_8FBr$	0.6250
S-7	H		76.36	122	$C_{39}H_{39}O_7N_8F$	0.5869
S-8	Cl		63.77	126	$C_{39}H_{38}O_7N_8FCl$	0.6216
S-9	Br		64.19	141	$C_{39}H_{38}O_7N_8FBr$	0.6390

4.1.1. Synthesis of 3-(4'-sulfadiazinyl) isatin

Equimolar (0.06 mol) quantities of isatin and sulfadiazine were dissolved in warm ethanol in the presence of two drops of glacial acetic acid and refluxed on a water bath for 1 h. The reaction mixture was allowed to stand for 24 h at room temperature. The precipitated product

was collected and recrystallized from ethanol. Yield: 81.1%; m.p.: 240 °C; IR (KBr): 3 400, 1 650, 1 320, 1 156, 800, 700 cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ (ppm): 6.5–7.5 (m, 8H, Ar–H), 8.5 (m, 3H, pyrimidinyl), 10.4 (s, 1H, NH), 11.2 (s, 1H, SO_2NH).

4.1.2. Synthesis of 3-(4'-sulfadoximino) isatin

Equimolar quantities of isatin and sulfadoxine were refluxed for 4 h in ethanol in the presence of 2–3 drops of glacial acetic acid. The contents were then cooled at room temperature and the product was obtained by filtration, washed with ethanol, dried and recrystallized from an ethanol and chloroform mixture. Yield: 89.72%; m.p.: 196 °C; IR (KBr): 3 230, 1 642, 1 579 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ (ppm) 6.75–7.55 (m, 9H, Ar–H), 10.1 (s, 1H, NH), 11.0 (s, 1H, SO_2NH).

4.1.3. Synthesis of 3-[4'-amino-5'-(3',4',5'-trimethoxybenzyl)pyrimidinyl]imino isatin

Isatin and trimethoprim in equimolar quantities were dissolved in ethanol containing 1 mL of glacial acetic acid. The reaction mixture was refluxed for 4 h and set aside. The resulting solid was washed with dilute ethanol, dried and recrystallized from an ethanol and chloroform mixture. Yield: 96%; m.p.: 185 °C; IR (KBr): 3 300, 3 050, 1 660, 1 620, 1 580 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ (ppm): 3.2 (s, 2H, CH_2), 3.7 (s, 9H, -OCH_3), 5.6 (s, 2H, NH_2), 6.8–7.1 (m, 7H, Ar–H), 10.6 (s, 1H, SO_2NH).

4.1.4. General procedure for the preparation of Mannich bases

To a solution of 1-ethyl-6-fluoro-1,4-dihydro-7-piperazin-1-yl-4-oxo quinoline-3-carboxylic acid (norfloxacin, 0.02 mol) in glacial acetic acid (50 mL), was added isatin derivatives (0.02 mol) and 37% formalin (1 mL). The reaction mixture was refluxed over a water bath for 1–3 h. TLC control indicated that norfloxacin had disappeared almost completely. The reaction mixture was concentrated to approximately half of the initial volume, and the resulting precipitate was recrystallized from a mixture of DMF and water.

4.1.5. 1-Ethyl-6-fluoro-1,4-dihydro-4-oxo-7[[N^4-(3'-sulfadiazinimino-1'-isatinyl)methyl]N^1-piperazinyl]-3-quinoline carboxylic acid **S-1**

Yield: 76.56%; m.p.:125 °C; IR (KBr): 2 850, 1 730, 1 640, 1 620, 1 325, 1 155 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ (ppm): 1.78 (t, 3H, CH_3 of C_2H_5), 3.7–4.1 (m, 8H, piperazine-H), 4.88 (q, 2H, CH_2 of C_2H_5), 5.2 (s, 2H, -NCH_2N-), 6.7–8.5 (m, 13H, Ar–H), 9.32 (s, 1H, C_2–H), 11.3 (s, 1H, -SO_2NH); calculated for $C_{35}H_{31}O_6N_8F$: C, 61.9; H, 4.6; N, 16.5. Found: C, 61.74; H, 4.30; N, 16.41%.

4.1.6. 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7[[N^4-(3'-sulfadoximino-1'-isatinyl)methyl]N^1-piperazinyl]-3-quinoline carboxylic acid **S-4**

Yield: 64.07%; m.p.: 119 °C; IR (KBr); 2 840, 1 730, 1 620, 1 583, 1 167, 1 596 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ (ppm): 1.60 (t, 3H, CH_3 of C_2H_5), 3.2 (s, 6H, OCH_3), 3.8–4.0 (m, 8H, piperazine-H), 4.34 (q, 2H, CH_2 of C_2H_5), 4.9 (s, 2H, -NCH_2N-), 6.6–8.35 (m, 11H, Ar–H), 8.64 (s, 1H, C_2–H), 10.9 (s, 1H, -SO_2NH); calculated for $C_{37}H_{35}O_8N_8F$: C, 60.15; H, 5.19; N, 15.16; found: C, 60.11; H, 5.10; N, 14.92%.

4.1.7. 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7[[N^4-[3'-(4'-amino-5'-trimethoxybenzylpyrimidin-2'-yl)imino-1'-isatinyl]methyl]N^1-piperazinyl]-3-quinoline carboxylic acid **S-7**

Yield: 76.36%; m.p.: 122 °C; IR (KBr): 3 010, 2 860, 2 840, 1 730, 1 616, 1 506, 1 236, 1 129 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ (ppm): 1.60 (t, 3H, CH_3 of C_2H_5), 3.3 (s, 2H, -CCH_2C-), 3.54 (s, 9H, -OCH_3), 3.7–4.1 (m, 8H, piperazine-H), 4.2 (q, 2H, CH_2 of C_2H_5), 5.1 (s, 2H, -NCH_2N-), 5.8 (s, 2H, NH_2), 6.58–8.56 (m, 9H, Ar–H), 8.6 (s, 1H, C_2–H); calculated for $C_{39}H_{39}O_7N_8F$: C, 62.39; H, 5.23; N, 14.92; found: C, 62.12; H, 5.36; N, 14.73%.

4.2. Biology

4.2.1. In vitro antibacterial screening

Compounds were evaluated for their in vitro antibacterial activity against 28 pathogenic bacteria procured from the Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University. The agar dilution method [13] was performed using Mueller-Hinton agar (Hi-Media) medium. Suspensions of each microorganism were prepared to contain approximately 10^6 colony forming units (cfu/mL) and applied to plates with serially diluted compounds in DMF to be tested and incubated at 37 °C overnight (approx. 18–20 h). The minimum inhibitory concentration (MIC) was considered to be the lowest concentration that completely inhibited growth on agar plates, disregarding a single colony or a faint haze caused by the inoculum.

4.2.2. In vivo antibacterial studies (mouse protection test)

The in vivo antibacterial activity of the test compounds was determined in CF-strain male mice (20–25 g body weight, six per group). The mice were infected intraperitoneally with a suspension containing an amount of the indicated organism slightly greater than its lethal dose 100 (LD_{100}). The mice were treated orally (p.o.) with a specific amount of the test compound administered at 1 and 4 h after infection. ED_{50} values were calculated by

interpolation among survival rates in each group after a week. They express the total dose of compound (mg/kg) required to protect 50% of the mice from an experimentally induced lethal systemic infection of the indicated organism.

4.2.3. In vitro antifungal screening

The compounds were evaluated for their in vitro antifungal activity against *Candida albicans, Aspergillus niger, C. neoformans, Microsporum audouinii, Trichophyton mentagrophytes, Epidermophyton floccosum, Microsporum gypsum* and *H. capsulatum* using an agar dilution method with Sabouraud's dextrose agar (Hi-Media). Suspensions of each micro-organism were prepared to contain 10^5 cfu/mL and applied to agar plates which have been serially diluted with compounds to be tested in DMF. The plates were incubated at 25 °C for 48–72 h and MICs were determined.

4.2.4. Anti-HIV screening

The compounds were tested for anti-HIV activity against replication of HIV-1 (III B) in MT-4 cells. The MT-4 cells were grown in RPMI-1640 DM (Dutch modification) medium (Flow lab, Irvine Scotland), supplemented with 10% (v/v) heat-inactivated calf serum and 20 μg/mL gentamicin (E. Merck, Darmstadt, Germany). HIV-1 (III B) [14] were obtained from the culture supernatant of HIV-1 infected MT-4 cell lines [15] and the virus stocks were stored at –70 °C until used. Anti-HIV assays were carried out in microtitre plates filled with 100 μL of medium and 25μL volumes of compounds in triplicate so as to allow simultaneous evaluation of their effects on HIV and mock infected cells. Fifty microlitres of HIV at 100 $CCID_{50}$ medium were added to either the HIV infected or mock infected part of the microtitre tray. The cell cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. Five days after infection the viability of mock and HIV-infected cells were examined spectrophotometrically by the MTT method [16].

4.2.5. Acute toxicity on oral administration in mice

A suspension of each compound in a 0.5% CMC was administered orally to CF-strain male mice (20–25 g body weight, six per group). Seven days later, LD_{50} values were determined by using the Weil method [17].

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