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New insight for fluoroquinophenoxazine derivatives as possibly new potent topoisomerase I inhibitor

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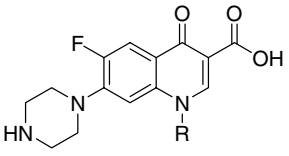
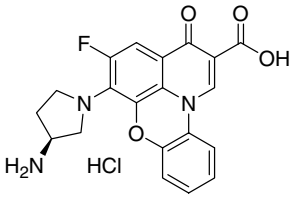
Abstract—Fluoroquinolones, represented by ciproxacin and norfloxacin, are well-known clinical antimicrobial agents, and their phenyl ring expanded quinophenoxazines are reported as possible antitumor active compounds. These quinophenoxazines are known to inhibit DNA topoisomerase II essential for cell replication cycle. But there were no reports for topoisomerase I inhibition study for these compounds. In this report, we have prepared a few quinophenoxazine analogues and tested their topoisomerases I and II inhibitory activities and cytotoxicity. From the result, we found that quinophenoxazine analogues possessed strong topoisomerase I inhibitory capacity as well as topoisomerase II inhibition. Among the compounds prepared, A-62176 analogues showed strong topoisomerases I and II inhibitory activities. Interestingly, compound **8** missing the 3-aminopyrrolidine moiety at C2 position has similar potent inhibitory capacity against topoisomerases I and II at higher concentrations (20 and 10 μM, respectively). But compound **8** inhibited topoisomerase I function more selectively at lower concentration, 2 μM. Our observation might strongly implicate that fluoroquinophenoxazines can be developed as efficient topoisomerase I inhibitor with the elaborate modification.

Fluoroquinolones, represented by ciproxacin (**1a**) and norfloxacin (**1b**),[1] are well-known clinical antimicrobial agents. The pharmacological activities of this class of compounds are known to be originated from DNA gyrase and topoisomerase IV inhibition.[2] The similarity between bacterial and eukaryotic DNA topoisomerase II mechanistic aspects had stimulated many researchers to prepare and test phenyl ring expanded fluoroquinophenoxazines as possible new antitumor agents.[3] Among the compounds, A-62176 (**2**)[3a] reported by Abbott researchers showed good inhibitory activity against human and murine cancer cell lines. Based on this information, several types of phenyl ring expanded fluoroquinophenoxazines have been synthesized and tested for the development of prospective anticancer agents.[4] Since it has been known that the fluoroquinophenoxazines are topoisomerase II inhibitor,[5] most of the biological mechanistic tests are devoted to topoisomerase II inhibition pathways. However, there are no reports against topoisomerase I for this class of compounds.



1a (Ciproxacin) R = Cyclopropyl
1b (Norfloxacin) R = Ethyl



2 (A-62176)

In this report, we have prepared six fluoroquinophenoxazine analogues with (*S*)-A-62176. And we have tested their topoisomerase I inhibitory activities to determine the possibility if fluoroquinophenoxazines can be exploitable as new topoisomerase I inhibitors. We also tested topoisomerase II inhibitory activity to compare the selectivity between two topoisomerases.

The structures of prepared compounds are depicted in Figure 1. In order to synthesize these compounds we

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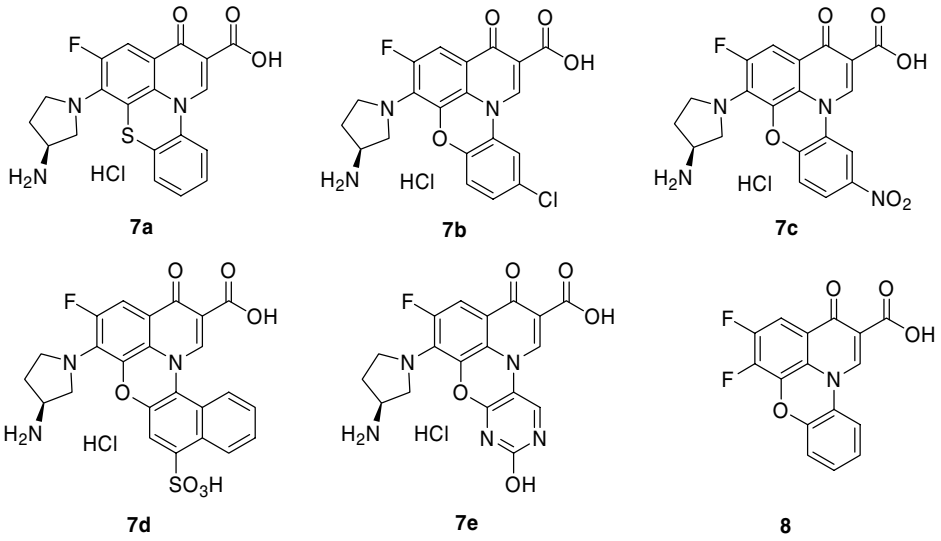


Figure 1. Structures prepared.

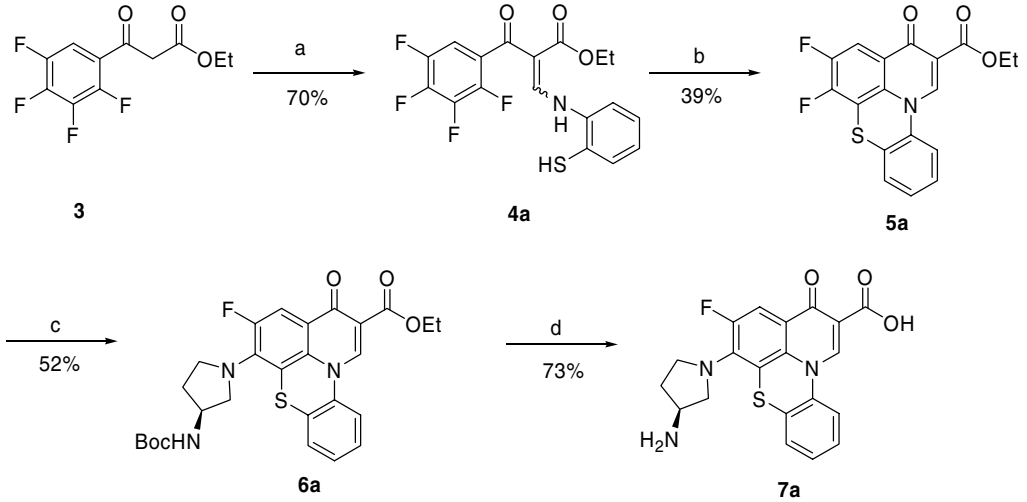
employed methods previously reported[3a,6] (see Scheme 1). The key starting compounds, *O*-aminoaromatic alcohol or thiol, were used with commercially available ones. The in situ coupling of *O*-aminothiophenol and product of ethyl 2,3,4,5-tetrafluorobenzoylacetate (**3**) and triethyl orthoformate in acetic anhydride afforded **4a**. Double ring cyclization under $NaHCO_3$ basic condition and subsequent regioselective nucleophilic substitution reaction at C-2 position with 3(*S*)-(*tert*-butoxycarbonylamino)pyrrolidine produced **6a**. Final hydrolysis of ethyl carboxylate and removal of protected group from amino group produced **7a**. With this procedure, six fluoroquinophenoxazine analogues including A-62176 were prepared. Among these compounds, five new quinophenoxazines **7a**–**e** and two known ones, **8**[6] and **A-62176**,[4c] were prepared. The spectral data for the compounds, **7a**–**e**, are indicated in the reference section.[7]

Topoisomerases I and II tests were conducted with supercoiled pBR322 plasmid DNA according to the literature methods.[8] First, in the topoisomerase II inhibition results showed that five compounds except **7d** and **7e** have inhibited the enzyme activity potently compared

Table 1. Topoisomerase I and II inhibitory activities of prepared compounds

Compounds	Topo I (% Inhibition)		Topo II (% Inhibition)	
	2 μM	20 μM	2 μM	10 μM
Camptothecin	63.3	84.1	ND	ND
Etoposide	ND	ND	0.7	40.9
7a	45.1	97.2	57.0	91.7
7b	87.9	86.1	89.0	88.6
7c	65.7	95.3	26.0	91.6
7d	39.1	25.0	1.9	24.7
7e	32.4	0.0	0.0	28.8
A-62176	88.9	90.2	89.1	89.4
8	53.5	96.6	0.5	82.4

The values of % inhibition are means from three independent experiments.



Scheme 1. General synthetic method for the target compounds. (a) 1. $CH(OEt)_3$, Ac_2O; 2. 2-Aminothiophenol; (b) $NaHCO_3$; (c) 3-*t*-Boc-(*S*)-aminopyrrolidine; (d) 1. NaOH; 2. HCl.

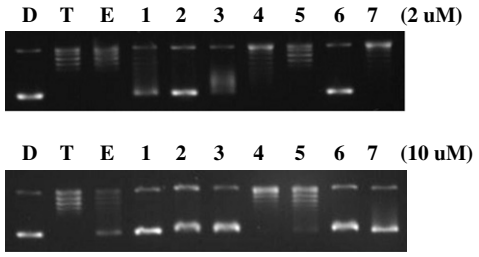


Figure 2. Topoisomerase II inhibitory activities of prepared compounds. Compounds were examined in a final concentration of 2 and 10 μM, respectively. Lane D: pBR322 only, Lane T: pBR322 + Topo II, Lane E: pBR322 + Topo II + etoposide, Lanes 1–7: pBR322 + Topo II + compounds in designated concentrations (compounds **7a**–**e**, **A-62176**, and **8**).

to etoposide used as positive control. (Table 1 and Fig. 2) These active quinophenoxazine analogues exhibited much higher inhibition efficiency than etoposide at 2 μM concentration. But, compounds **7d** and **7e** possessing fused naphthalenyl and pyrimidyl moieties have not shown good biological activity. Interestingly, compound **8** missing 3(*S*)-aminopyrrolidine group still revealed good enzyme inhibitory activity at higher concentration, 20 μM, compared with A-62176. Based on this result, we suspected that the phenyl ring fusion into benzoxazine core is important to exert topoisomerase II inhibition

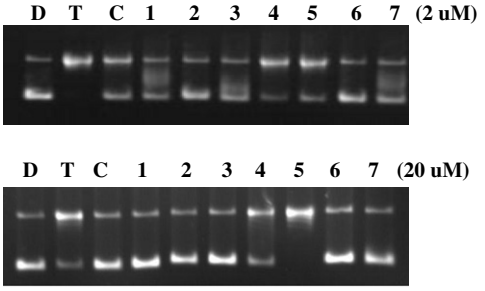


Figure 3. Topoisomerase I inhibitory activities of prepared compounds. Compounds were examined in a final concentration of 2 and 20 μM, respectively. Lane D: pBR322 only, Lane T: pBR322 + Topo I, Lane C: pBR322 + Topo I + camptothecin, Lanes 1–7: pBR322 + Topo I + compounds in designated concentrations (Compounds **7a**–**e**, **A-62176**, and **8**).

activity. Next, we have conducted the topoisomerase I inhibition test for the compounds. and the results are surprising. Basically, topoisomerase I inhibitory activity pattern was quite similar to that of topoisomerase II. Five compounds except **7d** and **7e** showed comparable activity with camptothecin at the range of tested concentrations, 2 and 20 μM (Table 1 and Fig. 3). The test compounds possessing structural similarity with A-62176 exhibited potent inhibitory activities but others, **7d** and **7e**, did not. These two topoisomerases inhibition results are quite interesting and impressive since the quinophenoxazine core can play the role as a dual repressor against topoisomerases I and II action. Compound **8** also exhibited enzyme inhibition activity comparable to A-62176 at lower concentration, 2 μM. But compound **8** had not shown inhibitory action against topoisomerase II at the same concentration. This information might suggest the possibility that modification of 3(*S*)-aminopyrrolidine group attached to quinophenoxazine core controls the selectivity between the topoisomerases I and II. But the clear factors and mechanism of compound **8** for selective inhibition on the topoisomerase I function over II at lower concentration should be studied in more detail.

With these results, we pursued the cytotoxicity test[9] to correlate the topoisomerases inhibition ability and cytotoxic properties for the tested compounds. Disappointingly, the compounds were not anticancer active ones as potent as positive controls and even less than A-62176 (Table 2).

In conclusion, five compounds except **7d** and **7e** showed very potent inhibitory capacity against topoisomerases I and II. These data, however, were not parallel with the cytotoxic activities, which are common in biological activity study in vitro.[10] Our findings reflect that fluoroquinophenoxazizne analogues are potent inhibitor against topoisomerase I as well as topoisomerase II. In addition, more elaborate consideration of the SAR for these analogues could provide better information for the development of new class of topoisomerase I inhibitors. For the clear SAR paradigm of the quinophenoxazine analogues as selective topoisomerase I, more compounds should be tested and analyzed.

Table 2. Cytotoxicities of prepared compounds (**7a**–**e**, **A-62176**, and **8**) against various cancer cells

	IC_{50}[a] (μM)				
Compound/cells	DU 145	HCT 116	HeLa	MDA-MB231	HL-60
Adriamycin	1.57 ± 0.06	1.10 ± 0.07	3.09 ± 0.32	1.39 ± 0.07	0.72 ± 0.04
Camptothecin	3.20 ± 0.40	1.00 ± 0.02	1.08 ± 0.67	4.16 ± 0.33	0.013 ± 0.002
Etoposide	29.79 ± 1.98	15.11 ± 0.87	18.24 ± 1.21	16.25 ± 1.84	1.44 ± 0.08
7a	19.30 ± 1.98	2.41 ± 0.26	5.08 ± 1.36	19.17 ± 1.17	11.51 ± 0.51
7b	17.60 ± 0.97	2.56 ± 0.03	2.01 ± 0.16	19.34 ± 0.88	1.63 ± 0.13
7c	13.83 ± 1.13	7.45 ± 0.78	17.74 ± 1.57	19.76 ± 0.23	9.93 ± 0.19
7d	>50	>50	>50	>50	>50
7e	>50	>50	>50	>50	>50
A-62176	1.82 ± 0.23	0.87 ± 0.20	2.33 ± 0.08	4.34 ± 0.10	2.40 ± 0.08
8	>50	6.30 ± 0.17	>50	>50	>50

[a] Each data point represents the mean ± SD from three different experiments performed in triplicate. Cell lines used are DU 145, human prostate tumor; HCT 116, human colon tumor; HeLa, human cervix tumor; MDA-MB231, human breast tumor; HL-60, human myeloid leukemic tumor.

Acknowledgment

This work was financially supported by Catholic University of Daegu, Korea.

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7. Compound **7a**: ^{1}H NMR (400 MHz, CD_3OD + DMSO-d_6) δ 2.04–2.15 (m, 1H), 2.48–2.57 (m, 1H), 3.43–3.50 (m, 2H), 3.58 (dd, J = 14.8, 8.8Hz, 1H), 3.71 (dd, J = 10.4, 6.4 Hz, 1H), 3.99–4.04 (m, 1H), 7.38–7.48 (m, 3H), 7.63 (d, J = 7.6 Hz, 1H), 7.80 (d, J = 12.4 Hz, 1H), 9.26 (s, 1H); ^{13}C NMR (100 MHz, CD_3OD + DMSO-d_6) 29.9, 48.7, 50.4, 54.1, 108.8 ($J_{C\text{-}F}$ = 23.8 Hz), 109.6, 120.6, 123.6, 125.2 ($J_{C\text{-}F}$ = 8.1 Hz), 125.8, 128.2, 128.6, 129.4, 134.4, 135.3 ($J_{C\text{-}F}$ = 14.9 Hz), 135.4, 143.7, 158.8 ($J_{C\text{-}F}$ = 252.1 Hz), 167.1, 177.6.
   Compound **7b**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.98–2.03 (m, 1H), 2.21–2.25 (m, 1H), 3.71–3.74 (m, 2H), 3.81–3.83 (m, 2H), 3.91–3.94 (m, 1H), 7.29 (d, J = 8.8 Hz, 1H), 7.40 (d, J = 8.8 Hz, 1H), 7.43 (d, J = 14.0 Hz, 1H), 8.20 (s, 1H), 9.10 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) 30.1, 49.9, 50.0, 55.1, 105.2 ($J_{C\text{-}F}$ = 24.5 Hz), 108.2, 116.8 ($J_{C\text{-}F}$ = 8.1 Hz), 117.7, 119.9, 125.3, 126.4, 129.5 ($J_{C\text{-}F}$ = 14.2 Hz), 129.6, 129.8, 134.4 ($J_{C\text{-}F}$ = 8.9 Hz), 139.5, 143.2, 154.1 ($J_{C\text{-}F}$ = 246.9 Hz), 165.9, 176.5.
   Compound **7c**: ^{1}H NMR (400 MHz, CD_3OD) δ 2.12–2.20 (m, 1H), 2.42–2.48 (m, 1H), 3.83–3.88 (m, 2H), 4.01–4.05 (m, 2H), 4.07–4.10 (m, 1H), 7.40 (d, J = 8.8 Hz, 1H), 7.44 (d, J = 13.2 Hz, 1H), 8.19 (d, J = 8.8 Hz, 1H), 8.55 (s, 1H), 9.06 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) 30.0, 49.9, 50.0, 55.1, 105.7, 108.5, 114.1, 116.4 ($J_{C\text{-}F}$ = 9.7 Hz), 119.3, 125.0, 125.3, 126.4, 129.9 ($J_{C\text{-}F}$ = 16.4 Hz), 133.6, 134.4, 140.2, 144.5, 149.3, 154.0 ($J_{C\text{-}F}$ = 246.1 Hz), 165.8, 176.4.
   Compound **7d**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.94–1.98 (m, 1H), 2.19–2.24 (m, 1H), 3.67–3.70 (m, 2H), 3.83–3.87 (m, 2H), 3.93–3.97 (m, 1H), 7.41 (d, J = 14.0 Hz, 1H), 7.55 (dd, J = 7.6, 7.2 Hz, 1H), 7.61 (dd, J = 8.0, 7.2 Hz, 1H), 7.83 (s, 1H), 8.08 (d, J = 7.6 Hz, 1H), 8.08 (d, J = 7.6 Hz, 1H), 8.09 (br s, 3H), 8.90 (d, J = 8.0 Hz, 1H), 9.12 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) 30.0, 49.9, 50.0, 55.2, 105.5, 107.6,116.2, 117.1 ($J_{C\text{-}F}$ = 8.2 Hz), 119.7, 120.7, 125.2, 126.2, 128.5, 128.6, 129.3, 129.5, 129.6, 134.8 ($J_{C\text{-}F}$ = 8.9 Hz), 144.4, 144.6, 146.6, 153.4 ($J_{C\text{-}F}$ = 246.2 Hz), 166.2, 176.5.
   Compound **7e**: ^{1}H NMR (400 MHz, CD_3OD) δ 2.06–2.15 (m, 1H), 2.34–2.42 (m, 1H), 3.70–3.77 (m, 2H), 3.91–4.02 (m, 3H), 7.88 (d, J = 14.0 Hz, 1H), 8.05 (s, 1H), 8.56 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) 30.4, 50.2, 51.2, 55.8, 108.3, 108.6, 109.0, 114.1, 118.5 ($J_{C\text{-}F}$ = 8.1 Hz), 121.2, 141.3, 152.3, 153.7, 162.7, 168.3, 177.9. Other peaks were not observed due to low concentration of this compound.
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9. **Cytotoxicity assay**: Cancer cells were cultured according to the supplier's instructions. Cells were seeded in 96-well plates at a density of 2–4 × 10^4 cells per well and incubated overnight in 0.1 mL of media supplied with 10% Fetal Bovine Serum (Hyclone, USA) in 5% CO_2 incubator at 37 °C. On day 2, culture medium in each well was exchanged with 0.1 mL aliquots of medium containing graded concentrations of compounds. On day 4, each well was added with 5 μL of the cell counting kit-8 solution

(Dojindo, Japan) and then incubated for additional 4 h under the same condition. The absorbance of each well was determined by an Automatic Elisa Reader System (Bio-Rad 3550) at a 450 nm wavelength. For determination of the IC_{50} values, the absorbance readings at 450 nm were fitted to the four-parameter logistic equation. The compounds of adriamycin, etoposide, and camptothecin were purchased from Sigma and used as positive controls.

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