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Synthesis and biological evaluation of a class of quinolone triazoles as potential antimicrobial agents and their interactions with calf thymus DNA

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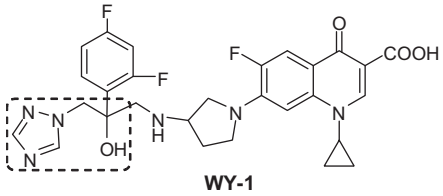
ABSTRACT

A novel series of quinolone triazoles were synthesized and characterized by IR, NMR, MS and HRMS spectra. All the newly prepared compounds were screened for their antimicrobial activities against seven bacteria and four fungi. Bioactive assay manifested that most of new compounds exhibited good or even stronger antibacterial and antifungal activities against the tested strains including multi-drug resistant MRSA in comparison with reference drugs Norfloxacin, Chloromycin and Fluconazole. The preliminary interactive investigations of compound **6b** with calf thymus DNA by fluorescence and UV–vis spectroscopic methods revealed that compound **6b** could effectively intercalate DNA to form compound **6b**–DNA complex which might block DNA replication and thus exert its antimicrobial activities.

Quinolones are one of the most widely prescribed antimicrobial agents as they are generally well tolerated with excellent safety profile, favorable pharmacokinetic characteristics and broad antibacterial spectrum against genitourinary infections and common respiratory tract pathogens. It is well known that quinolones targeting bacterial type II topoisomerases can bind with the enzyme–DNA binary complex to form ternary complexes, thereby blocking DNA replication and leading to bacterial cells' death.[1] Since the first generation of quinolone drugs was found to be active in the early 1960s, four generations of quinolones have been clinically used in succession, such as Nalidixicacid, Pipemidicacid, Lomefloxacin, Moxifloxacin and so on.[2]

Nevertheless, their extensive use and narrow antibacterial spectrum especially low activity against Gram-positive pathogens, including *staphylococci*, *streptococci*, and *enterococci* have greatly affected the therapeutic efficacy in clinic.[3] Furthermore, their shortcomings including phototoxicity, lipid peroxidation, photohemolysis and bad water-solubility also limit the administrable mode.[4] This situation stimulates the further structural modification of quinolones. Especially the N-1 position and benzene ring in quinolones are adaptable sites for regulating physicochemical property and greatly influencing the antimicrobial potency, spectrum and safety.[5] Two successful examples are Ciprofloxacin and Norfloxacin with cyclopropyl and ethyl groups respectively at the N-1 position, which display different bioavailability and antibacterial spectrum. Moreover, the substituents on benzene ring of quinolones, such as Ciprofloxacin and Moxifloxacin, can also make contributions to their bioactivities.[6]



WY-1

Our recent work indicated that the introduction of triazolyl ethanol moiety in the C-7 side chain of ciprofloxacin could effectively inhibit the growth of all the tested bacteria and fungi. The clinafloxacin derivative **WY-1** with a 2,4-difluorophenyl group displayed more potent antimicrobial efficacy (MIC = 0.25–1 μg/mL) than its positive control.[7] When the triazole ring was introduced into N-1 position of quinolones, the resulting quinolone triazoles also showed great potential to treat the drug-resistant bacterial infections.[8] These results clearly pointed out that the hybrids of antibacterial quinolones with antifungal triazole moiety could not only remarkably enhance the antimicrobial activities, but also broaden the antimicrobial spectrum.

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In view of the above observations,[9] herein we incorporated triazolyl ethanol fragment into the N-1 position of quinolones and changed different substituents on benzene ring of quinolones to generate a novel class of quinolone triazoles. The prepared hybrids might be expected to exhibit potential against bacterial and fungal strains including drug-resistant microorganism. Therefore, their antibacterial and antifungal activities for all newly synthesized compounds were evaluated in vitro against three Gram-positive bacteria including *Methicillin-Resistant Staphylococcus aureus* N315 (MRSA), four Gram-negative bacteria and four fungi. The preliminary antimicrobial mechanism was investigated by evaluating the interaction of the prepared highly active compound with calf thymus DNA.

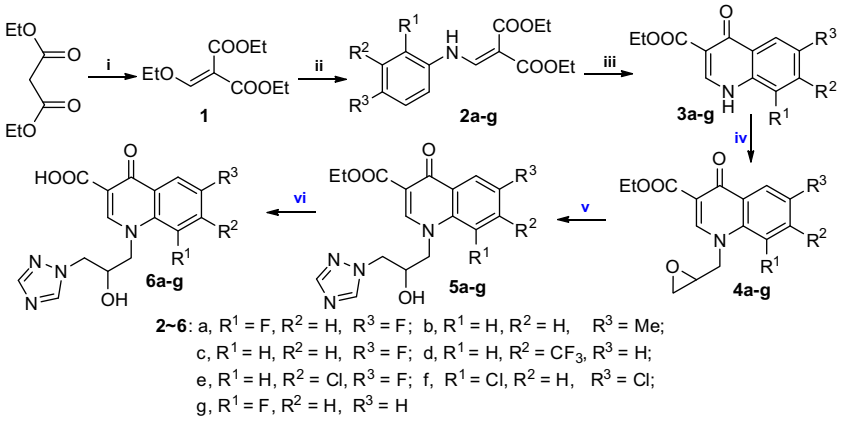
The target quinolone triazole compounds were synthesized according to the synthetic route outlined in Scheme 1. Commercially available diethyl malonate was reacted with triethoxymethane in the presence of anhydrous zinc chloride to produce diethyl 2-(ethoxymethylene) malonate **1** in an almost quantitative yield of 97%, and then compound **1** was further treated with a series of substituted phenylamines in ethanol to afford diethyl 2-((phenylamino)methylene)malonate derivatives **2a**–**g**, which were cyclized in oxydibenzene under reflux to give the desired quinolones **3a**–**g**.[10] The latter was further N-alkylated by commercial 2-(chloromethyl)oxirane to yield racemic ethyl 1-(oxiran-2-ylmethyl) quinoline-3-carboxylate derivatives **4a**–**g** in high yields (82–91%), subsequently the epoxy ring was opened by 1,2,4-triazole in ethanol using sodium bicarbonate as base to produce racemates **5a**–**g**, and then were further hydrolyzed in water by 3% sodium hydroxide at 100 °C to afford the corresponding target quinolone triazoles **6a**–**g** with excellent yields. All the new compounds were confirmed by ^{1}H NMR, ^{13}C NMR, IR, MS and HRMS spectra.

All synthesized quinolone triazoles were evaluated in vitro for their antimicrobial activities against three Gram-positive bacteria (*Micrococcus luteus* ATCC 4698, MRSA and *Staphylococcus aureus* ATCC25923), four Gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli* DH52, *Shigella dysenteriae*, and *Eberthella typhosa*) and four fungi (*Candida utilis*, *Aspergillus flavus*, *Beer yeast*, and *Candida albicans*) by two folds serial dilution technique recommended by National Committee for Clinical Laboratory Standards (NCCLS) with the positive control of clinically antimicrobial drugs Norfloxacin, Chloromycin and Fluconazole. The antimicrobial tests were carried out for three times. The obtained results as depicted in Table 1 revealed that quinolone triazoles **5a–g** and **6a–g** could effectively inhibit the growth of most tested bacterial strains, while compounds **4a**–**g** exhibited good activities against all the tested fungal strains except *C. utilis*. Almost all the new compounds (MIC = 0.5–8 μg/mL) showed comparable or even superior activity against *E. typhosa* to standard drugs Norfloxacin (4 μg/mL) and Chloromycin (32 μg/mL). Especially compound **6d** with a trifluoromethyl group at the 7-position of quinolone backbone gave stronger antibacterial efficacies and broader bioactive spectrum than Norfloxacin and Chloromycin with quite low MIC values.

It was known that MRSA was one of the most virulent organisms that showed severe multi-drug resistance to numerous currently available agents. Excitingly, all the new compounds except for compounds **5a**, **5d**, **5g**, **6e** and **6g** could effectively inhibit the growth of MRSA at the concentrations of 0.5–16 μg/mL, which were more effective than clinical Chloromycin (MIC = 16 μg/mL). It was specially noticed that compounds **5f**, **6c** and **6d** with MIC values of 1 μg/mL were 8- and 16-fold more active than Norfloxacin (MIC = 8 μg/mL) and Chloromycin respectively. Especially 7-trifluoromethyl intermediate **4d** (MIC = 0.5 μg/mL), as a new potential anti-MRSA agent, was 16- and 32-fold more active than both of standard drugs Norfloxacin and Chloromycin respectively.

The antifungal evaluation in vitro revealed that most of the newly prepared compounds exhibited completely different results in comparison with their antibacterial activities. Quinolones **4a**–**g** with oxiran-2-ylmethyl group at *N*-1 position showed good inhibitory efficiency against all the tested fungal strains except for *C. utilis*, which gave comparable or superior inhibitory potency to the first-line antifungal drug Fluconazole. Especially compounds **4a**, **4c**, **4d**, **4f** and **4g** could efficiently inhibit the growth of *A. flavus* strain in vitro with MIC values ranging from 0.5 to 1 μg/mL, which were 256-fold more potent than Fluconazole (MIC = 256 μg/mL). Surprisingly, the 6-methyl quinolone triazole **6b** displayed not only good antibacterial activities against all the tested bacterial strains, but also excellent antifungal activities against *A. flavus*, *B. yeast*, and *C. albicans* in low inhibitory concentrations (MIC = 0.5–4 μg/mL). Based on these observations and analysis, compound **6b** might act different target site from standard drugs Fluconazole (cytochrome P_{450}) and Norfloxacin (topoisomerase IV), which suggested this compound might inhibit bacterial and fungal strains growth by different mechanisms of action.

Generally the substituents in quinolone B ring could affect antimicrobial potency. Compound **4b** with electron-donating methyl substituent exhibited poor activities against *M. luteus*, *E. coli DH52* and *S. dysenteriae* (MIC = 256, 128 and 128 μg/mL, respectively). On the other hand, electron-withdrawing halogen groups especially fluorine moiety in 6-, 7- and 8-position of quinolones exerted great influences on antibacterial activities. Trifluoromethyl quinolone triazole **6d** and 7-chloro-6-fluoro quinolone **6e** showed



Scheme 1. Reagents and conditions: (i) triethoxymethane, acetic anhydride, $ZnCl_2$, N_2, reflux, 4 h; (ii) substituted phenylamines, EtOH, reflux, 2 h; (iii) Ph_2O, reflux, 1 h; (iv) 2-(chloromethyl)oxirane, K_2CO_3, 120 °C, 3 h; (v) triazole, K_2CO_3, EtOH, reflux, 2 h; (vi) 3% NaOH, 100 °C, 2 h.

Table 1
In vitro antimicrobial data as MIC (μg/mL) for compounds **4**–**6**

Compds	Fungi				Gram-positive bacteria			Gram-negative bacteria			
	C. utilis	*A. flavus*	*B. yeast*	*C. albicans*	*M. luteus*	MRSA	*S. aureus*	*P. aeruginosa*	*E. coli* DH52	*S. dysenteriae*	*E. typhosa*
4a	>512	1	64	4	2	8	16	64	128	>512	2
4b	256	16	64	2	32	16	256	32	128	128	2
4c	256	1	2	16	512	4	32	128	64	64	8
4d	>512	0.5	8	4	128	0.5	256	64	64	256	2
4e	>512	8	4	64	64	8	128	32	64	128	8
4f	>512	0.5	8	2	>512	2	1	0.5	32	>512	2
4g	>512	0.5	4	1	>512	2	2	256	16	512	2
5a	>512	64	512	64	2	32	1	4	8	32	0.5
5b	>512	256	64	128	16	2	64	2	4	4	4
5c	64	512	512	32	8	8	32	16	16	64	1
5d	128	8	64	64	2	32	16	1	32	32	0.5
5e	>512	16	512	8	4	4	64	4	32	32	1
5f	>512	0.5	32	512	8	1	1	4	4	128	4
5g	>512	512	32	32	2	128	64	32	8	8	0.5
6a	>512	256	512	8	2	8	2	2	2	16	1
6b	>512	0.5	4	2	16	8	32	16	32	32	8
6c	>512	512	512	64	32	1	128	8	32	0.5	1
6d	>512	512	256	1	2	1	1	8	8	1	4
6e	>512	128	256	16	16	64	64	2	8	64	4
6f	>512	256	128	32	64	4	64	8	64	4	16
6g	>512	128	256	16	8	64	32	8	4	1	64
Chloromycin	>512	>512	>512	>512	8	16	16	32	32	32	32
Norfloxacin	>512	>512	>512	>512	2	8	0.5	1	16	4	4
Fluconazole	8	256	16	1	>512	>512	>512	>512	>512	>512	>512

C. utilis, *Candida utilis*; *A. flavus*, *Aspergillus flavus*; *B. yeast*, *Beer yeast*; *C. albicans*, *Candida albicans*; *M. luteus*, *Micrococcus luteus* ATCC 4698; MRSA, *Methicillin-Resistant Staphylococcus aureus* N315; *S. aureus*, *Staphylococcus aureus* ATCC25923; *P. aeruginosa*, *Pseudomonas aeruginosa*; *E. coli* DH52, *Escherichia coli*; *S. dysenteriae*, *Shigella dysenteriae*; *E. typhosa*, *Eberthella typhosa*.

comparable or even superior activities to Chloromycin and Norfloxacin against all the tested strains. The results suggested that the introduction of hydroxyl and triazolyl moieties as well as electron-donating groups could improve the antibacterial efficiencies to some extent.

Thus a preliminary antimicrobial mechanism was explored by evaluating the interaction between the strongest active compound **6b** and calf thymus deoxyribonucleic acid (DNA). DNA is the informational molecule encoding the genetic instructions used in the development and function of almost all known living organisms. In recent years, DNA as a therapeutic target has attracted considerable attention in biomedical science. These binding studies with small molecules are important and helpful for developing novel and more efficient drugs. Calf thymus DNA was selected as DNA model because of its medical importance, low cost and ready availability properties. Therefore, in order to investigate the possible mechanism of antimicrobial action, the binding studies between the highly active compound **6b** and calf thymus DNA on molecular level were carried out by UV–vis spectroscopic and fluorescent methods.[11]

In absorption spectroscopy, hypochromism and hyperchromism are very important spectral features to distinguish the change of DNA double-helical structure. Due to the strong interaction between the electronic states of intercalating chromophore and that of the DNA base, the observed large hypochromism strongly suggests a close proximity of the aromatic chromophore to the DNA bases.[12] With a fixed concentration of DNA, UV–vis absorption spectra were recorded with increasing amount of compound **6b**. As shown in Figure 1, UV–vis spectra displayed that the maximum absorption peak of DNA at 260 nm exhibited proportional increase and slight blue shift along with the increasing concentration of compound **6b**. Meanwhile the phenomenon, that the absorption value of simply sum of free DNA and free compound **6b** was a little greater than the measured value of DNA–compound **6b** complex, was observed in the inset of Figure 1. This meant that a weak hypochromic effect existed between DNA and compound **6b**. Moreover

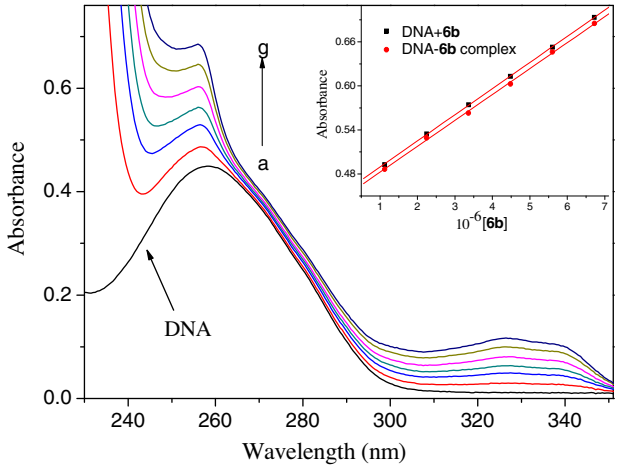


Figure 1. UV absorption spectra of DNA with different concentrations of compound **6b** (pH 7.4, T = 288 K). Inset: comparison of absorption at 260 nm between the DNA–compound **6b** complex and the sum values of free DNA and free compound **6b**. c(DNA) = 7.61×10^{-5} mol/L, and c(compound **6b**) = 0–6.72×10^{-6} mol/L for curves a–g respectively at increment 1.12×10^{-6}.

the intercalation of the aromatic chromophore of compound **6b** into the helix and the strong overlap of π–$\pi*$ states of the large π-conjugated system with the electronic states of DNA bases were consistent with the observed spectral changes. Based on the variations in these absorption spectra, equation (1) could be used to calculate the intrinsic binding constant (K):

$$\frac{A^0}{A-A^0} = \frac{\xi_C}{\xi_{D-C}-\xi_C} + \frac{\xi_C}{\xi_{D-C}-\xi_C} \times \frac{1}{K[6b]} \quad (1)$$

A^0 and A represent the absorbance of DNA in the absence and presence of compound **6b** at 260 nm, ξ_C and ξ_{D-C} were the absorption coefficients of compound **6b** and DNA–compound **6b** complex respectively. The plot of $A^0/(A-A^0)$ versus 1/[compound **6b**] was

constructed by using the absorption titration data and linear fitting, yielding the binding constant, $K = 1.43 \times 103$ L/mol, $R = 0.998$, SD = 0.17 (R is the correlation coefficient. SD is standard deviation).

To further understand the interaction between compound **6b** and DNA, the absorption spectra of competitive interaction of compound **6b** were investigated. Compared with other common probes, Neutral Red (NR) showed lower toxicity, higher stability and convenient application. Furthermore, it has been sufficiently demonstrated by spectrophotometric and electrochemical techniques that NR can bind to DNA by an intercalative mode.[13] Therefore NR was employed as a spectral probe to investigate the binding mode of compound **6b** with DNA in this work. The absorption peak of the NR around 272 nm gradually decreased by increasing the concentration of DNA, which suggested the formation of new DNA–NR complex (Supplementary data). Figure 2 displayed the absorption spectra of a competitive binding between NR and compound **6b** with DNA. As the gradually increasing concentration of compound **6b**, an apparent intensity increase was observed around 272 nm. Compared with the absorption around 272 nm of NR–DNA complex, the absorbance at the same wavelength (inset of Fig. 2) exhibited the reverse process. In addition to that, the absorbance at 255 and 327 nm and extensive broadening were also observed in the spectra. These various spectral changes were consistent with the intercalation of compound **6b** into DNA by substituting for NR in the DNA–NR complex.

Measurement of fluorescence was also carried out to prove the interaction between compound **6b** and DNA. With a fixed concentration of DNA, fluorescence spectra were recorded with increasing amount of compound **6b**. The fluorescence intensity of the peak around 376 nm increased gradually with increasing the concentration of compound **6b**. Furthermore, the fluorescence spectrum of compound **6b** in the absence of DNA was also recorded under the same conditions (Supporting Information). Figure 3 showed that there were apparent differences about max fluorescence intensity between them. At the same concentration of compound **6b**, the system max fluorescence intensity in absence of DNA was higher than the system in presence of DNA, which meant the binding of compound **6b** with DNA helix was found to strongly quench the fluorescence of compound **6b**. From the experimental results, it was concluded that the chromophore of compound **6b** could intercalate into the DNA helix.

Steady-state quenching experiments by highly negatively charged quenchers can provide further information about the

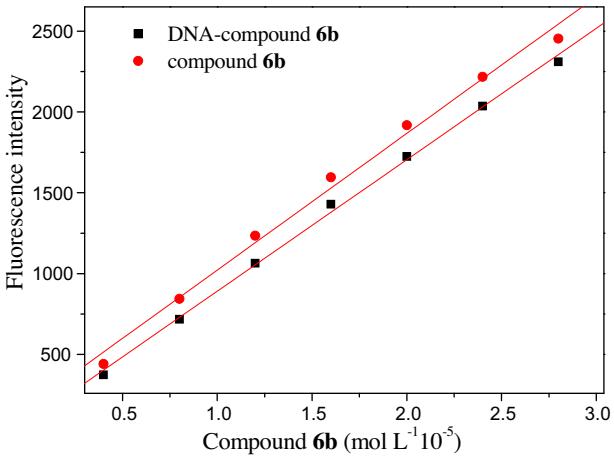


Figure 3. Relative fluorescence intensity for compound **6b** at different concentrations with or without DNA. c(DNA) = 7.18×10^{-6} mol/L, and c(compound **6b**) = 0–8.0×10^{-5} mol/L for curves a–t, respectively at increment 0.4×10^{-5}.

binding of molecule with DNA. To investigate the binding mechanism, iodide quenching studies were introduced in this work. Generally, small molecules have three modes *via* diverse non-covalent interactions to bind with double-helix DNA including electrostatic interaction with the anionic sugar phosphate backbone of DNA, groove binding with the DNA groove, and intercalation with DNA base pairs. Among the three bonding modes, the intercalative binding and groove binding are the most effective interactions mode to block DNA replication in comparison to electrostatic interaction.[14]

In the presence of anionic quencher, DNA base pairs around the intercalators can hinder the accessibility of fluorescent probe to quencher. While the electrostatic repelling between DNA phosphate backbone and anionic quenchers will provide protection for the intercalated species. Therefore, if the binding mode is intercalation, the molecule should be protected from being quenched by anionic quencher. The value of quenching constants (K_{SV}) of the intercalative bound molecule should be lower than that of the molecule bound to DNA by groove binding.[15] Negatively charged I^- ion was selected for this purpose. The values of K_{SV} of compound **6b** by I^- ion in the absence and presence of DNA were calculated to be 7.65 L/mol ($R = 0.997$, SD = 0.05) and 6.53 L/mol ($R = 0.998$, SD = 0.04), respectively (Fig. 4). The results suggested that the

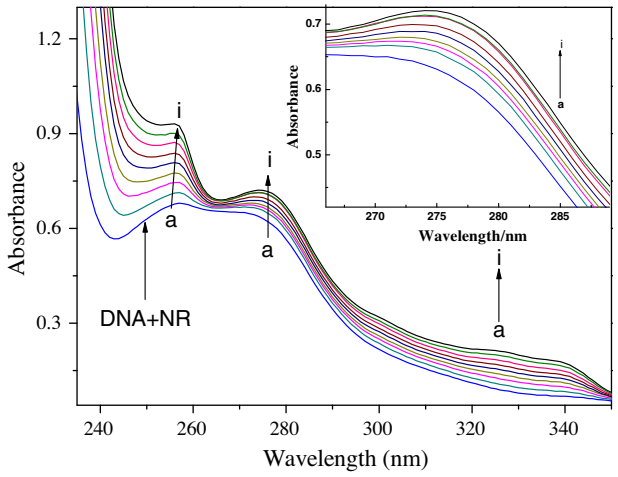


Figure 2. UV absorption spectra of competitive reaction between compound **6b** and NR with DNA. c(DNA) = 6.04×10^{-5} mol/L, c(NR) = 2×10^{-5} mol/L, and c(compound **6b**) = 0–8.0×10^{-5} mol/L for curves a–i, respectively at increment 1.0×10^{-5}.

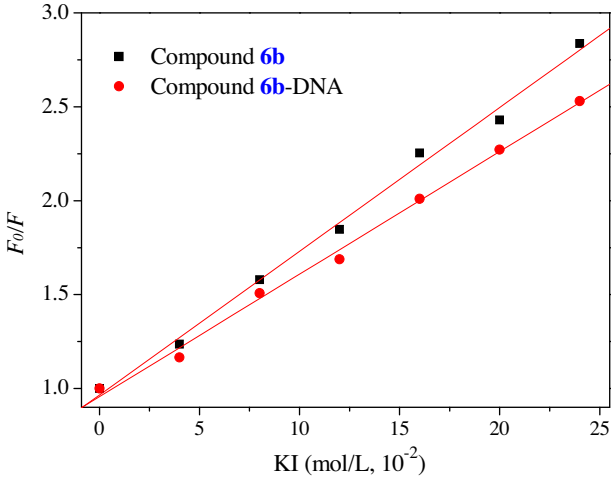


Figure 4. Fluorescence quenching plot of compound **6b** by KI in the absence and presence of DNA (pH 7.4, $T = 288$ K). c(compound **6b**) = 1×10^{-5} mol/L, and c(DNA) = 7.18×10^{-5} mol/L

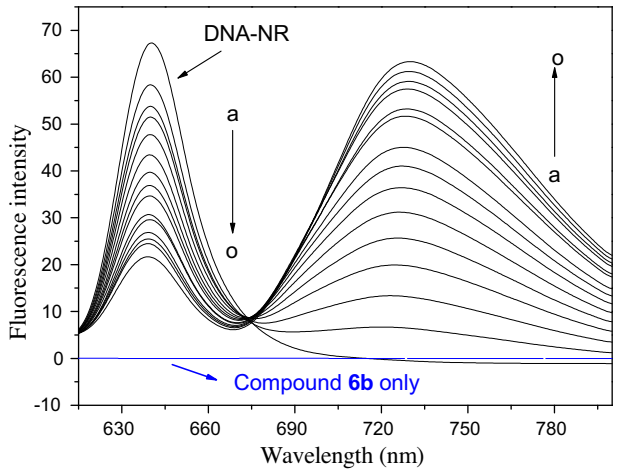


Figure 5. Fluorescence spectra of DNA–NR in the presence and absence of compound **6b** at different concentrations (pH 7.4, $T = 288$ K). c(DNA) = 7.18 × 10^{-6} mol/L, and c(compound **6b**) = 0–6.0 × 10^{-5} mol/L for curves a–o respectively at increment 0.4 × 10^{-5}. Blue line shows the emission spectrum of compound **6b** only.

binding mode of compound **6b** with DNA was of intercalation nature.

In further examination the NR was introduced as a fluorescence probe to investigate the binding characteristics of compound **6b** with DNA.[16] Figure 5 showed the emission spectra of the DNA–NR system in the presence and absence of compound **6b**. The only emission spectrum of **6b** indicated that it did not posses significant fluorescence features, thus the effect of compound **6b** on fluorescence of DNA-NR complex would be negligible. When the concentration of compound **6b** was increasing gradually, a remarkable fluorescence decrease in DNA–NR system was observed around 640 nm. Furthermore, an obvious intensity increase was observed around 726 nm. This phenomenon suggested that compound **6b** could compete with NR for the intercalation sites in DNA and lead to a significant decrease at 640 nm and a remarkable increase at 726 nm. These results meant that compound **6b** could intercalate into the helix of DNA.

In conclusion, a novel class of quinolone triazoles were designed and synthesized in good yields via an easy, convenient and efficient synthetic route. All the new compounds were characterized by ^{1}H NMR, ^{13}C NMR, MS, IR and HRMS spectra.[17] The in vitro antimicrobial activities of these quinolone triazoles and their intermediates were evaluated against seven bacterial and four fungal strains.[18] The biological results revealed that most of the newly synthesized compounds exhibited good antibacterial and antifungal activities against most of the tested strains in comparison to the reference drugs Chloromycin, Norfloxacin and Fluconazole. Quinolone triazoles **6d** and **6e** showed significant inhibition against all tested bacterial strains with low inhibitory concentrations in range of 1–64 μg/mL. Intermediates **4a**–**g** gave better antifungal activities against all the tested fungal strains except *C. utilis* than Fluconazole. Especially compound **6b** showed potent antibacterial and antifungal efficacy against all the tested strains. The specific interaction of compound **6b** with DNA was studied by fluorescence and UV–vis absorption spectroscopy. The experimental results displayed that compound **6b** could intercalate DNA to form compound **6b**–DNA complex which might further block DNA replication to exert their powerful antibacterial and antifungal activities. Further researches, including the quenching mechanism of fluorescence of DNA by the compound **6b**, the thermodynamic parameters, binding sites, the in vivo bioactive evaluation along with toxicity investigation and the effect factors on antimicrobial activities such as other heterocyclic azole rings (benzotriazole, imidazole, benzimidazole and their derivatives) as well as other substituents on skeleton quinolone ring are now in progress. All these will be discussed in future paper.

Acknowledgments

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17. *Experimental:* melting points were recorded on X-6 melting point apparatus and were uncorrected. TLC analysis was done using pre-coated silica gel plates. FT-IR spectra were carried out on Bruker RFS100/S spectrophotometer (Bio-Rad, Cambridge, MA, USA) using KBr pellets in the 400–4000 cm^{-1} range. NMR spectra were recorded on a Bruker AV 300 spectrometer using TMS as an internal standard. The chemical shifts were reported in parts per million (ppm), the coupling constants (*J*) are expressed in hertz (Hz) and singlet (s), doublet (d) and triplet (t), broad (br) as well as multiplet (m). The mass spectra (MS) were recorded on LCMS–2010A and the high-resolution mass spectra (HRMS) were recorded on an IonSpec FT-ICR mass spectrometer with ESI resource.
Synthesis of ethyl 6,8-difluoro-1-(oxiran-2-ylmethyl)-4-oxo-1,4-dihydroquinoline-3-carboxylate (**4a**). A mixture of compound **3** (0.253 g, 1.0 mmol) and potassium carbonate (0.151 g, 1.2 mmol) was stirred in 2-(chloromethyl)oxirane (15 mL) under reflux for 3 h. After the reaction was completed, the mixture was cooled to room temperature, the excess 2-(chloromethyl) oxirane was evaporated under reduced pressure, and water was added. The residue was extracted with chloroform (3 × 20 mL), the combined organic phase was dried over anhydrous sodium sulfate, and then the residue was purified by column chromatography (eluent, chloroform/methanol 70:1, V/V) to give the desired compound **4a** (0.294 g) as light yellow solid. Yield: 94.7%; mp >250 °C. IR (KBr, cm^{-1}): 3131 (Ar-H), 3016 (=C–H), 2988, 2902 (CH_2, CH_3), 1683, 1641 (C=O), 1609, 1559, 1493 (aromatic frame), 1370, 1237, 1085, 1028, 981, 852; ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.51 (s, 1H, C-2 quinolone), 7.84–7.93 (m, 1H, C-5 quinolone), 7.79–7.81 (m, 1H, C-7

quinolone), 4.85–4.90 (m, 1H, O-C*H*), 4.45–4.50 (m, 2H, N-CH_2), 4.26 (q, 2H, OCH_2CH$_3$), 2.54–2.55 (m 2H, OCH_2) 1.29 (t, *J* = 7.1 Hz, 3H, OCH$_2$CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 170.95, 164.50, 160.24, 156.97, 154.61, 152.22, 151.25, 131.92, 130.43, 110.32, 60.37, 57.21, 50.26, 44.87, 14.71; MS (ESI): *m*/*z* 310 [M+H]$^+$; HRMS (ESI) calcd for $C_{15}H_{13}F_2NO_4$ [M+H]$^+$, 310.0813; found, 310.0811.

Synthesis of ethyl 6,8-difluoro-1-(2-hydroxy-3-(1H-1,2,4-triazol-1-yl)propyl)-4-oxo-1,4-dihydroquinoline-3- carboxylate (**5a**). To a stirred suspension of potassium carbonate (0.151 g, 1.2 mmol) in ethanol was added 1,2,4-triazole (0.691 g, 1.0 mmol). The mixture was stirred at 60 °C for 1 h. The reaction was cooled to room temperature, compound **4a** (0.309 g, 1.0 mmol) was added at the room temperature and stirred for 2 h under reflux. After the reaction came to end, solvent was evaporated and the residue was extracted with chloroform (3 × 20 mL). The combined organic extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude product was purified by column chromatography (eluent, chloroform/methanol 30:1, V/V) to afford the compound **5a** (0.408 g) as yellow solid. Yield: 85.2%; mp >250 °C. IR (KBr, cm^{-1}): 3443 (O–H), 3129 (Ar-H), 3016 (=C–H), 2989, 2902 (CH$_2$, CH$_3$), 1771, 1676 (C=O), 1614, 1564, 1508 (aromatic frame), 1318, 1254, 1099, 1021, 961, 853; ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.58 (s, 2H, C-2 quinolone and triazole 3-*H*), 8.07 (s, 1H, triazole 5-*H*), 7.41–7.44 (m, 1H, C-5 quinolone), 7.24–7.28 (m, 1H, C-7 quinolone), 5.66 (m, 1H, O*H*), 4.97 (m, 1H, O-C*H*), 4.19–4.69 (m, 6H, –CH_2–), 1.26 (t, *J* = 7.1 Hz, 3H, OCH$_2$CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 172.23, 164.73, 155.22, 153.65, 153.14, 152.78, 150.26, 145.42, 131.23, 128.57, 123.16, 110.06, 68.23, 60.90, 60.35, 52.58, 14.73. MS (ESI): *m*/*z* 379 [M+H]$^+$; HRMS (ESI) calcd for $C_{17}H_{16}F_2N_4O_4$ [M+H]$^+$, 379.1140; found, 379.1142.

Synthesis of 6,8-difluoro-1-(2-hydroxy-3-(1H-1,2,4-triazol-1-yl)propyl)-4-oxo-1,4-dihydroquinoline-3- carboxylic acid (**6a**) To a stirred solution of sodium hydroxide (3%, 20 mL) was added compound **5a**. The mixture was stirred at 100 °C for 2 h. After the reaction was completed, the mixture was treated with formic acid to adjust pH to 7, and then the suspension was filtered and washed with water for three times to give the target compound **6a** (0.282 g) as light yellow solid. Yield: 80.5%; mp >250 °C; IR (KBr, cm^{-1}): 3420 (O–H), 3130 (Ar-H), 3016 (=C–H), 2996, 2903 (CH$_2$, CH$_3$), 1730, 1665 (C=O), 1630, 1565, 1507 (aromatic frame), 1323, 1296, 1112, 1019, 997, 893, 804; ^{1}H NMR (300 MHz, DMSO-d_6): δ 14.95 (s, 1H, COO*H*), 8.95 (s, 1H triazole 3-*H*), 8.57 (s, 1H, C-2 quinolone), 8.07 (s, 1H, triazole 5-*H*), 7.59–7.62 (m, 1H, C-5 quinolone), 7.46–7.49 (m, 1H, C-7 quinolone), 5.77 (m, 1H, O*H*), 5.05 (br, 1H, O-C*H*), 4.36–4.84(m, 4H, –CH_2–); ^{13}C NMR (75 MHz, DMSO-d_6): δ 173.21, 164.64, 155.31, 153.69, 153.01, 152.43, 150.21, 145.32, 131.23, 128.57, 123.16, 110.06, 68.21, 60.35, 52.58, MS (ESI): *m*/*z* 351 [M+H]$^+$; HRMS (ESI) calcd for $C_{15}H_{12}F_2N_4O_4$ [M+H]$^+$, 351.0827; found, 351.0830.

18. National Committee for Clinical Laboratory Standards Approved standard Document. M27-A2, Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts, National Committee for Clinical Laboratory Standards, Wayne, PA, 2002.