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Original article

Synthesis and topoisomerase inhibitory activities of novel aza-analogues of flavones[1]

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Abstract – A series of aza-flavones (3-hydroxy-2-phenyl-4-quinolones) were designed and synthesized as inhibitors of bacterial DNA-gyrase and mammalian topoisomerase II. Structure activity relationships of the compounds against each of the enzymes are discussed. © Elsevier, Paris

aza-flavones / 3-hydroxy-2-phenyl-4-quinolones / DNA-gyrase / topoisomerase II

1. Introduction

DNA topoisomerases catalyse the topological interconversions of DNA molecules which are required for several essential processes in DNA metabolism, including replication, recombination, transcription, and chromosome separation at mitosis [1]. These enzymes have therefore served as targets for several useful antitumour [2] and antibacterial agents [3]. Both prokaryotic and eukaryotic topoisomerase II inhibitors, developed as either antibacterial or antitumour agents, respectively, share a similar mechanism of action. The formation of a ternary complex (DNA::topoisomerase II::drug) leading to the "cleavable-complex" is required for the activity of the inhibitors [4–8]. Specific binding sites and strand specificity for covalent catalysis leading to formation of the ternary drug complex are unique, even though both enzymes share a common tyrosine for the covalent catalysis [9–13]. These differences afford the possibility of finding agents that inhibit only the prokaryotic topoisomerase II, such as the antibacterial 4-quinolones [14–16], or inhibitors of the mammalian topoisomerase II, such as the antitumour quinolones [15, 17–21].

Figure 1. Structures of flavones **1** and aza-analogues of flavones **2**.

In the process of searching for prokaryotic topoisomerase II (DNA gyrase) inhibitors, we recently identified a series of flavones **1** as bona fide DNA gyrase (bacterial type II topoisomerase) inhibitors by the supercoiling and cleavable complex assays. Some of the compounds (e.g. quercetin: IC_{50} = 3.3 μg/mL) were as potent as some of the currently marketed 4-quinolone antibacterials (e.g. ofloxacin: IC_{50} = 1.75 μg/mL) against the target enzyme [22]. This led us to synthesize a series of the hitherto unknown aza-analogues of flavones **2** *(figure 1)*. Since literature evidence indicated that some

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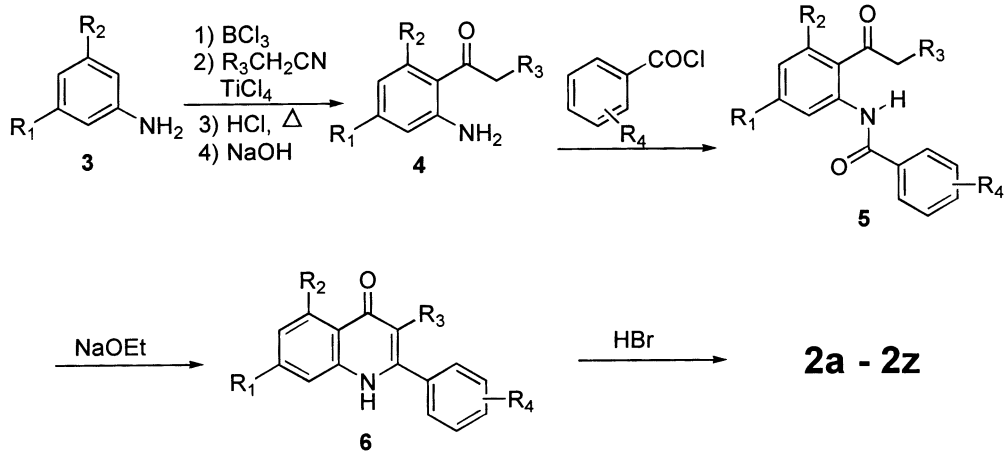


Figure 2. Synthesis of compounds **2a–z**.

flavones also had inhibitory activity against the mammalian DNA topoisomerase II [23] we decided to study our compounds against both enzymes. The synthesis and biological activities of these compounds were reported herein.

2. Chemistry

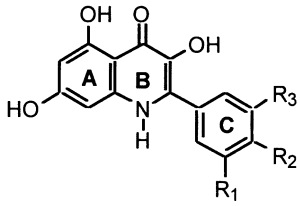
Literature searches indicated that the synthesis of 3-hydroxy-4-quinolones has been poorly investigated. The synthesis employed to these compounds are shown in *figure 2*. The ketones **4** were synthesized from the appropriately substituted anilines **3**, by regioselective acylation of the *ortho* positions with the corresponding acetonitriles [24, 25]. In most cases, titanium tetrachloride was used as a catalyst. Aluminium chloride and zirconium chloride can also be used in place of titanium chloride. For the synthesis of the non-symmetrical compounds **2w**, **2x**, **2y** and **2z**, 3-methoxy-aniline was converted to a mixture of the two regioisomers the 2- and 6-substituted derivatives. The ratio of the substitution of position 2 to that of position 6 was 5:7. The mixture was then separated by chromatography. N-acylation of **4** with substituted benzoylchlorides under standard conditions gave the amides **5**. The acylated derivatives **5** were then cyclized under pressure in the presence of sodium ethoxide to give the 3-methoxy-4-quinolones **6**. Demethylation of the various methoxy groups, where necessary, was accomplished in a single step either by refluxing with hydrobromic acid or heating with excess pyridine hydrochloride at 180–200 °C, which provided the hydroxy derivatives **2**. Compounds **2** are often hydroscopic and contain hydrobromic acid. This could be due to the presence of α- and β-hydroxy carbonyl as well as catechol moieties which can form chelates or intermolecular complexes with water and hydrobromic acid.

3. Results and discussion

The biological activities of compounds **2a–z**, ellipticine and nalidixic acid against the prokaryotic topoisomerase II (DNA-gyrase) and the eukaryotic topoisomerase II are shown in *tables I–V*. In general, the synthesized compounds possess activities against both bacterial DNA-gyrase and mammalian topoisomerase II. The compounds are usually more potent against topoisomerase II. The potencies of the compounds against the bacterial DNA-gyrase are in the range of the first generation quinolones, such as nalidixic acid (IC_{50} = 52 μg/mL). Compound **2a** (IC_{50} = 81 μg/mL), the aza analogue of quercetin is about 25-times less potent than its flavone counterpart (quercetin, IC_{50} = 3.3 μg/mL). The most potent compound **2b** (IC_{50} = 0.01 μg/mL) against topoisomerase II is 400-times more potent than the standard ellipticine (IC_{50} = 4.68 μg/mL).

The aza-flavones display distinct SAR against the two enzymes. When ring C is substituted with hydroxy groups *(table I)*, the compounds showed activities against both enzymes. While eliminating the hydroxy group does not abolish the activity against topoisomerase II (IC_{50} = 0.27 μg/mL), the compound (**2c**) was inactive against DNA-gyrase (IC_{50} > 500 μg/mL). Interestingly, the tri-

Table I. Importance of the hydroxyl groups on the C-ring.

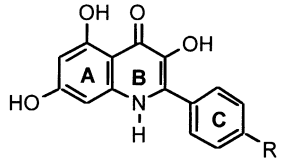


Compound	R_1	R_2	R_3	DNA-gyrase IC_{50} μg/mL	Topoisomerase II IC_{50} μg/mL
Ellipticine				> 500	4.68
Nalidixic Acid				52.0	> 500
2a	OH	OH	H	81.0	5.03
2b	OH	OH	OH	472	0.01
2c	H	H	H	> 500	0.27
2d	H	OH	H	200	1.94

hydroxy derivative **2b** showed high potency against topoisomerase II (IC_{50} = 0.01 μg/mL) but marginal activity against DNA-gyrase (IC_{50} = 472 μg/mL). This indicates that the binding sites of ring C for the two enzymes are different. Furthermore, the activity against topoisomerase II seems to be more sensitive to the modifications of ring C as illustrated in *table II*. Replacing the 4-hydroxy group with an *n*-butyl did not change the activity against DNA-gyrase but decreased the activity against topoiomerase II. Replacing the *n*-butyl with a *t*-butyl increased the gyrase activity slightly, but decreased the topoisomerase activity dramatically. We found that the phenolic protons on ring C are not required for the activities since the hydroxy groups of ring C can be replaced by halogens in most cases *(table III)*. These results suggest that the hydroxy groups do not serve as hydrogen-bonding donors in the binding to the enzymes. There is no clear evidence according to our data that the oxygen atoms of the hydroxy groups or the halogens may act as hydrogen-bonding acceptors though trifluoromethyl and bromine decrease or abolish the activities.

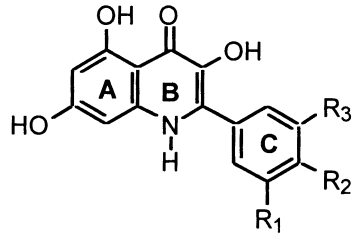
The hydroxy group on ring B is essential for the DNA-gyrase activity but not the topoisomerase activity as shown in *table IV*. This further indicates that the structure requirements for the two enzymes are different.

Table II. Replacement of the 4′-hydroxyl groups with other substituents.



Compound	R	DNA-gyrase IC_{50} μg/mL	Topoisomerase II IC_{50} μg/mL
2d	OH	200	1.94
2[e]	*n*-butyl	185	5.21
2f	*t*-butyl	67.0	45.9
2g	CO_2Et	314	27.0
2h	CO_2H	137	1.41
2i	NH_2	> 500	27.3
2j	CH_3	> 500	27.5

Table III. Replacement of C-ring hydroxyl groups with halogens.



Compound	R_1	R_2	R_3	DNA-gyrase IC_{50} μg/mL	Topoisomerase II IC_{50} μg/mL
2k	H	F	H	141	29.3
2l	F	H	F	131	0.12
2m	F	F	H	123	0.85
2n	H	Br	H	277	> 500
2o	Cl	H	Cl	159	5.07
2p	Cl	Cl	H	82.0	4.06
2q	CF_3	H	CF_3	103	30.0
2r	H	CF_3	H	> 500	51.3

Unlike ring B, the existence of the hydroxy groups on ring A is not critical to the activities against both enzymes, though the structure-activity relationships are once again different for each of the two enzymes *(table V)*. While the replacement of the hydroxy groups with fluorine did not affect the activities against both enzymes, the methyl substituted compound **2v** showed much higher potency against topoisomerase II (IC_{50} = 0.07 μg/mL) and no significant change against DNA-gyrase activity (IC_{50} = 113 μg/mL).

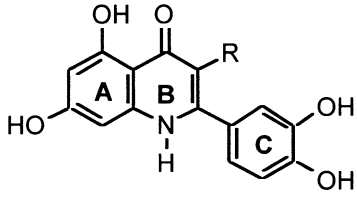
4. Conclusion

We have identified a series of novel aza analogues of flavones as inhibitors of bacterial DNA-gyrase and mammalian topoisomerase II. The compounds displayed divergent structure-activity relationships against each of the enzymes. While some compounds are more potent and selective against mammalian topoisomerase II than ellipticine, others showed comparable potency and selectivity against DNA gyrase as nalidixic acid.

5. Experimental protocols

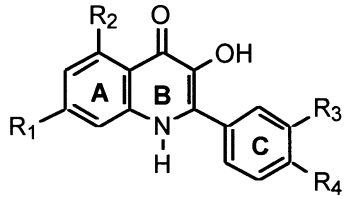
Melting points were determined on a Meltemp II apparatus and are uncorrected. Mass spectral data (chemical ionization technique) were performed by the analytical group at the R.W. Johnson Pharmaceutical Research Institute. All proton NMR spectra were recorded on a GE-300 spectrometer, and values are reported in ppm

Table IV. Importance of the 3-hydroxyl groups.



Compound	R_1	DNA-gyrase IC_{50} μg/mL	Topoisomerase II IC_{50} μg/mL
2a	OH	81.0	5.03
2s	H	> 500	0.29
2t	C_2H_5	> 500	21.0

Table V. Importance of the A-ring hydroxyl groups.



Compound	R_1	R_2	R_3	R_4	DNA-gyrase IC_{50} μg/mL	Topoisomerase II IC_{50} μg/mL
2a	OH	OH	OH	OH	81.0	5.03
2u	F	F	OH	OH	58.0	9.16
2v	CH_3	CH_3	OH	OH	113	0.07
2w	H	OH	OH	OH	> 500	2.80
2x	OH	H	OH	OH	133	1.14
2y	H	OH	H	H	> 500	75.7
2z	OH	H	H	H	> 500	4.76

from Me_4Si. Physical data of the final products are shown in *table VI*. The elemental analyses of all compounds are within the range of experimental error (± 0.4%).

Table VI. Physical data of the final products.

Compound	M.p.	Formula
2a	> 300	$C_{15}H_{11}NO_6 \cdot 0.5H_2O$
2b	> 300	$C_{15}H_{11}NO_6 \cdot HBr \cdot 0.5H_2O$
2c	175–177	$C_{15}H_{11}NO_4 \cdot HBr \cdot H_2O$
2d	> 300	$C_{15}H_{11}NO_5 \cdot HBr \cdot 0.5H_2O$
2e	137–139	$C_{15}H_{11}NO_5 \cdot 0.7HBr$
2f	191–193	$C_{19}H_{19}NO_4 \cdot 0.9HBr$
2g	185–189	$C_{18}H_{15}NO_6 \cdot HBr$
2h	> 300	$C_{16}H_{11}NO_6 \cdot HBr$
2i	> 300	$C_{15}H_{11}N_2O_4 \cdot 1.8HBr \cdot 0.6H_2O$
2j	261–263	$C_{15}H_{11}N_2O_4 \cdot HBr \cdot 0.8H_2O$
2k	232–233	$C_{15}H_{10}NO_4F \cdot HBr$
2l	213	$C_{15}H_9NO_4F_2 \cdot HBr \cdot 0.8H_2O$
2m	> 300	$C_{15}H_9NO_4F_2 \cdot HBr$
2n	215–217	$C_{15}H_{10}BrNO_4 \cdot HBr \cdot 0.7H_2O$
2o	> 300	$C_{15}H_9Cl_2NO_4 \cdot HBr \cdot H_2O$
2p	> 300	$C_{15}H_9Cl_2NO_4 \cdot HBr \cdot 0.8H_2O$
2q	298–299	$C_{17}H_9F_6NO_4 \cdot HBr \cdot 1.7H_2O$
2r	191–193	$C_{19}H_{19}NO_4 \cdot 0.9HBr$
2s	283–285	$C_{15}H_{11}NO_5 \cdot HBr \cdot 1.6H_2O$
2t	299–300	$C_{17}H_{15}NO_4 \cdot HBr$
2u	300–301	$C_{15}H_9F_2NO_4 \cdot HBr \cdot 0.8H_2O$
2v	255–256	$C_{17}H_{15}Cl_2NO_4 \cdot HBr \cdot 0.4H_2O$
2w	279–280	$C_{15}H_{11}NO_5 \cdot HBr \cdot 0.05H_2O$
2x	259–260	$C_{15}H_{11}NO_4 \cdot HBr \cdot 0.4H_2O$
2y	150–151	$C_{15}H_{11}NO_3 \cdot 0.8HBr$
2z	165–166	$C_{15}H_{11}NO_3 \cdot HBr \cdot 0.9H_2O$

5.1. 3,5-Dimethoxy-2-(methoxyacetyl)aniline (**4a**)*, a general procedure for the preparation of the ketones* **4**

To a cooled solution of 3,5-dimethoxyaniline (46.0 g, 0.30 mol) in benzene (360 mL) under nitrogen was added BCl_3 (1.0 M in CH_2Cl_2, 300 mL, 0.30 mol). The suspension was stirred in an ice bath for 45 min. Methoxyacetonitrile (23.5 g, 0.33 mol) was added, followed by the addition of $TiCl_4$, 1 M solution in CH_2Cl_2 (39 mL, 0.039 mol). The reaction mixture was heated at reflux for 22 h. The mixture was allowed to cool to 25 °C then 2 N HCl (600 mL) was slowly added. The resulting mixture was heated at reflux for 1 h and then cooled and basified with 5 M NaOH (240 mL). The product was extracted with EtOAc (5 × 500 mL). The organic layers were dried ($MgSO_4$) and concentrated in vacuo to give 40.2 g of a tan solid. Column chromatography on silica gel (CH_2Cl_2) gave 38.6 g (57%) of the title compound **4a** as an off-white solid, m.p. 129–131 °C, MS(CI, CH_4) MH^+ at 226. ^{1}H-NMR ($CDCl_3$): δ 6.60 (br s, 2H, N*H*$_2$), 5.72 (2s, 2H, Ar*H*), 4.53 (s, 2H, COC*H*$_2$OCH$_3$), 3.82 (s, 3H, ArOC*H*$_3$), 3.78 (s, 3H, ArOC*H*$_3$), 3.47 (s, 3H, COCH$_2$OC*H*$_3$). Anal. ($C_{11}H_{15}NO_4$) C, H, N.

5.2. N-(3,5-Dimethoxy-2-methoxyacetylphenyl)-3,4-dimethoxybenzamide (**5a**)*, a general procedure for the preparation of the ketone-amide* **5**

To a solution of 3,5-dimethoxy-2-(methoxyacetyl)-aniline (**4a**), (2.20 g, 9.77 mmol) in pyridine (75 mL) was added 3,4-dimethoxybenzoyl chloride (2.10 g, 10.5 mmol). The mixture was heated at reflux under nitrogen for 18 h then poured into ice (500 mL). The

precipitate was collected via filtration and air dried to give 3.61 g (95%) of the title compound **5a** as an off-white solid. The product was used to prepare 3,5,7-trimethoxy-2-(3,4-dimethoxyphenyl)-4-oxoquinoline (**6a**) without further purification.

5.3. 3,5,7-trimethoxy-2-(3,4-dimethoxyphenyl)-4-oxoquinoline (**6a**)*, a general procedure for the preparation of the aza-flavone precursors* **6a–6z**

Sodium ethoxide solution, prepared from sodium (0.30 g, 13.0 mmol) and ethanol (100 mL), was transfered into a Parr bomb containing a solution of N-(3,5-dimethoxy-2-methoxyacetylphenyl)-3,4-dimethoxy-benzamide (3.60 g, 9.24 mmol) in ethanol (absolute, 50 mL). The bomb was sealed and heated at 160–165 °C, 180 psi for 3 h. The reaction vessel was allowed to cool to room temperature and the solvent was removed under vacuum. Column chromatography (silica gel, 10% MeOH in EtOAc) gave 1.23 g (36%) of **6a** as a brown solid, m.p. 134–135 °C, MS (CI, CH_4): MH^+ at 372. ^{1}H-NMR (DMSO-d_6): δ 10.93 (br, s, 1H, N*H*), 7.22 (s, 1H, Ar*H*), 7.20 (d, *J* = 8 Hz, 1H, Ar*H*), 7.11 (d, *J* = 8 Hz, 1H, Ar*H*), 6.67 (s, 1H, Ar*H*), 6.26 (s, 1H, Ar*H*), 3.83 (s, 6H, ArOCH_3), 3.80 (s, 3H, ArOCH_3), 3.78 (s, 3H, ArOCH_3), 3.60 (s, 3H, ArOCH_3). Anal. ($C_{20}H_{21}NO_6$•0.50 H_2O) C, H, N.

5.4. 3,5,7-Trihydroxy-2-(3,4-dihydroxyphenyl)-4-oxoquinoline (**2a**)*, a general procedure for the preparation of the aza-flavones* **2a–2z**

A mixture of 3,5,7-trimethoxy-2-(3,4-dimethoxyphenyl)-4-oxoquinoline (0.70 g, 1.88 mmol) in aqueous 48% HBr (10 mL) was heated at reflux for 3.5 h. The reaction mixture was then cooled and the solvent was removed under vacuum. The residue was recrystallized from H_2O and EtOH and gave 0.35 g (49%) of the title compound **2a** as a dark yellow solid, m.p. > 300 °C, MS(CI, CH_4): MH^+ at 302. ^{1}H-NMR (DMSO-d_6): δ 11.32 (s, 1H, N*H*), 10.5–8.50 (br, s, 5H, ArO*H*), 7.20 (d, *J* = 2Hz, 1H, Ar*H*), 7.04 (dd, *J* = 8, 2Hz, 1H, Ar*H*), 6.87 (d, *J* = 8Hz, 1H, Ar*H*), 6.44 (d, *J* = 2Hz, 1H, Ar*H*), 5.97 (d, *J* = 2Hz, 1H, Ar*H*). Anal. ($C_{15}H_{11}NO_6$•0.50 H_2O) C, H, N.

6. Biology

6.1. DNA gyrase supercoiling inhibition assay [26]

0.25–0.40 μg pBR322 DNA (previously relaxed with topoisomerase I) was added to a reaction mixture composed of 1.4 mM ATP, 1.8 mM spermidine, 5 mM DTT, 0.14 mM Na_2EDTA, 6.5% glycerol, 24 mM KCl, 4 mM $MgCl_2$, 0.36 μg/ml bovine serum albumin (mol. biol. grade), and 35 mM Tris-HCl, pH 7.5. To this reaction, drug was added from DMSO-solubilized stocks (such that the final concentration of DMSO is < 3.5%), followed by 1 unit of gyrase holoenzyme (reconstituted Gyr A and Gyr B subunits). Each reaction was incubated for 30 min at 37 °C, and reactions were stopped by the addition of SDS (to 0.5%), Na_2EDTA (to 6 mM), and 5.35% glycerol containing 0.013% bromophenol blue (as tracking dye). The total reaction mixture was loaded onto either a 1% TAE or TBE agarose gel and was electrophoresed in a horizontal submarine apparatus to separate different DNA topoisomers. Gels were stained with EtBr, and visualized by Polaroid film 667 photography of fluoresced gels. The percent supercoiling was determined by the densitometric tracing (Collage™, Image Dynamics Corporation) of supercoiling versus relaxed DNA, normalized against no-drug control lanes.

6.2. Topoisomerase II assay [27, 28]

Each reaction contained an equal volume of Kinetoplast DNA (kDNA, TopoGen, Inc., Columbus, OH, 1.1–1.8 μg/reaction), 67 mM Tris-HCl, pH 8, 160 mM KCl, 13 mM $MgCl_2$, 0.7 mM ATP, 0.7 mM dithiothreitol, 0.06 μg BSA, with an equal volume of test compound, and 10 units of p170 human topoisomerase II enzyme (TopoGen, Inc.). Each reaction mixture was incubated for 5 min at 37 °C, and the reaction was stopped with 0.5% Sarkosyl, 0.0025% bromophenol blue, and 25% glycerol. Reaction samples were electrophoresed in 0.6% TBE-agarose gels at 30 mA. The gel was stained with EtBr, and visualized by Polaroid film 667 or 665 (Polaroid Corporation, Cambridge, MA) photography of fluoresced gels at 300 nm. The decatenated kDNA band was quantified by densitometric analysis (College™, Image Dynamics Corporation).

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