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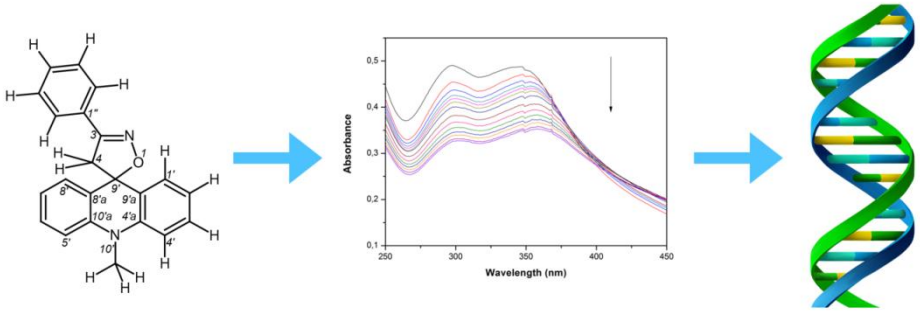
Graphical abstract



New spiro-acridine derivatives with DNA-binding and Topoisomerase I inhibition activity

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Abstract

Eight spiro-acridine derivatives containing the isoxazoline ring were synthesized and characterized using elemental analysis, IR, UV-Vis, and NMR measurements. Their interactions with calf thymus DNA were extensively studied by various spectroscopic techniques and gel electrophoresis. The UV-visible and CD measurements implied that these derivatives interact with calf thymus DNA through intercalation. The Stern-Volmer quenching constants were determined and ranged from 0.126×10^4 to 1.394×10^4 M^{-1}. A topoisomerase I inhibition assay was performed with the spiro-acridine derivatives.

Key words

spiro-acridine, isoxazoline, single crystal X-ray analysis, drug-DNA interaction, topoisomerase I inhibition

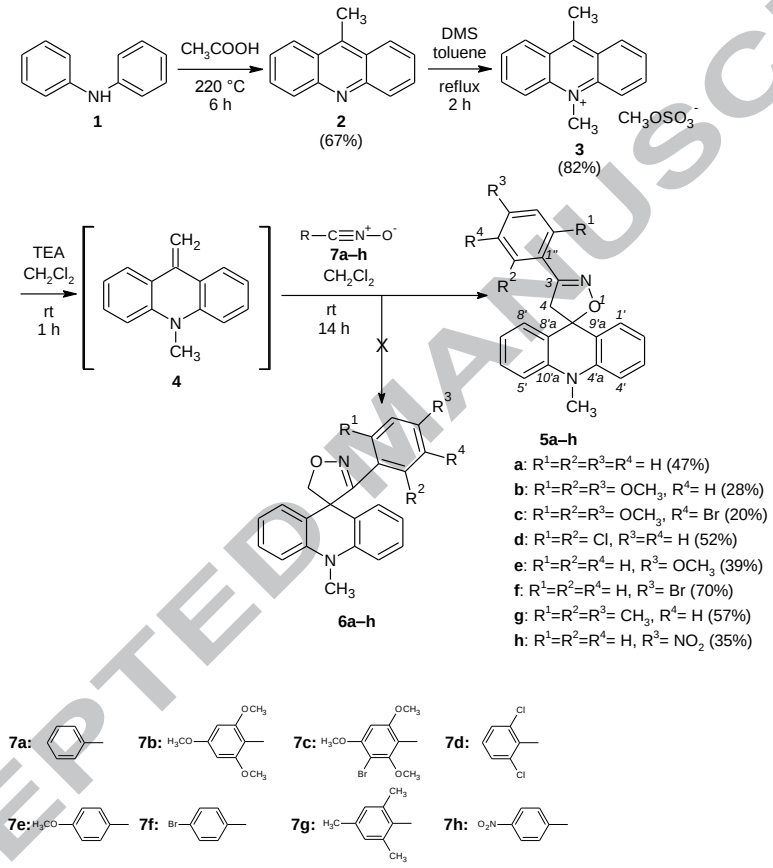
Acridine and its derivatives are interesting chemical families containing a planar aromatic chromophore which is able to bind into DNA by intercalation and thereby inhibit crucial classes of enzymes involved in the regulation of DNA, especially topoisomerases[1] and telomerases.[2] The strong fluorescence properties exhibited by acridine pharmacophores have led to their use in a number of fields of biological research, including as chemiluminescent agents, DNA intercalators, fluorescence reagents for the labeling of biomolecules, and chemical sensors in fluorescence spectroscopy.[3-5] Additionally, it has been reported that natural and non-natural isoxazolines and spirocyclic isoxazoline derivatives have shown promising antiproliferative effects on a variety of cancer cell lines.[6]

As part of our efforts to develop novel acridine derivatives possessing bioactive heterocyclic substituents, which are able to interfere with cellular processes, we have searched for new synthetic approaches for their preparation. The 1,3-dipolar cycloaddition reaction between nitrile oxides (NO) and alkenes is of considerable interest as an efficient way to prepare isoxazolines. For that reason, we have examined these reactions for the synthesis of new isoxazoline derivatives, which were screened for their DNA binding and topoisomerase I inhibition activities.

Herein, we report the three step synthesis of 10'-methyl-3-substituted-4*H*,10'*H*-spiro[acridine-9',5-[1,2]oxazoles] **5a–h** from the reactive acridine dipolarophile intermediate **4** which was synthesized according to Scheme 1. 9-Methylacridine (**2**) was converted into 9,10-dimethylacridinium methyl sulfate (**3**) using dimethyl sulfate according to a literature procedure,[7] followed by subsequent elimination to generate the exocyclic double bond of derivative **4**. Dipolarophile **4** was trapped with NO **7a–h** to afford the corresponding spiro-acridine products *via* addition to the exomethylenic double bond. NO **7a–h** were prepared according to literature procedures.[8] The treatment of compound **4** with NO **7a–h** yielded only a single regioisomer

5. The regioselectivity of these 1,3-dipolar cycloaddition reactions was not surprising as intermediate **4** represents a phenylogous enamine which is unambiguously polarized and regioselectively trapped by dipoles. Notably, in the reaction of dipolarophile **4** with NO **7b**, 3-brominated spiroisoxazoline analogue **5c** was also isolated in 20% yield when the NO reagent used was contaminated with 3-bromo-2,4,6-trimethoxybenzonitrile oxide (**7c**) which was partially formed as a by-product after the synthesis of **7b** from its bromooxime precursor.

The 1,3-dipolar cycloaddition reaction yields of compound **4** with NO compounds **7a–h** were low due to various reasons, including degradation of nitrile oxides in the reaction mixture, which occurred faster for nitrile oxides **7d, e, g–i** than for **7a–c, f**, as well as *in situ* release of reactive species from their precursors in the reaction mixture.



Scheme 1. Synthesis of 10'-methyl-3-substituted-4*H*,10'*H*-spiro[acridine-9',5-[1,2]oxazoles] **5a–h**.

In previous work[9,10] we found that the polarization of the dipolarophile CH=CH double bond as expressed by ^{13}C chemical shifts was useful for explaining the reaction regioselectivity. In this case, similar explanations were impossible due to the instability of dipolarophile **4**, which was generated *in situ*. The structural characterization of compounds **5a–h** was achieved using 1D and 2D NMR techniques. The ^{1}H, ^{13}C and ^{15}N NMR chemical shifts obtained for **5a**, as well as the $^{1}H,^{13}C$-HMBC and $^{1}H,^{15}N$-HMBC correlations, allowed unambiguous assignment of all atoms (Fig. 1) and was further confirmed by single crystal X-ray data (Fig. 2).

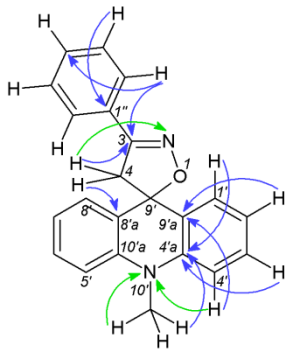


Figure 1. Selected $^{1}H,^{13}C$-HMBC (→) and $^{1}H,^{15}N$-HMBC (→) correlations in derivative **5a**.

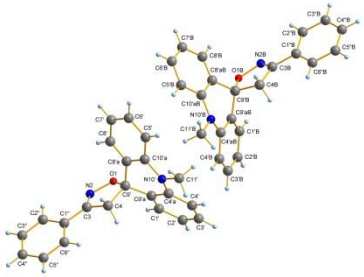


Figure 2. Single crystal X-ray structure of derivative **5a**.

Absorption spectroscopy is one of the most commonly employed instrumental techniques for studying DNA interactions with small ligands. The spectral titration of spiro-acridines **5a–h** was followed by monitoring the UV-Vis absorbance changes in the range of 240–450 nm. Upon addition of calf thymus DNA (CT DNA), the absorption bands showed significant hypochromism and a slight red shift (Table 1). The representative UV-vis spectrum of compound **5h** is shown in Figure 3. The observed hypochromism is caused by the DNA binding activity of the investigated compounds resulting from partial unwinding of the DNA double helix and consequent conformational changes.[11] A decrease of peak intensities with an absorbance reduction of approximately 14% to 35% from its initial value was observed. The hypochromism, due to a strong interaction between the electronic states of the intercalating chromophore and those of the DNA bases, was noted to be similar to previous reports,[12] suggesting the close proximity of the acridine ring and DNA. The presence of an isosbestic point at 400 nm indicated spectroscopically distinct chromophores, namely, free and bound species. Such behavior is generally associated with intercalation as a dominant binding mode.[13]

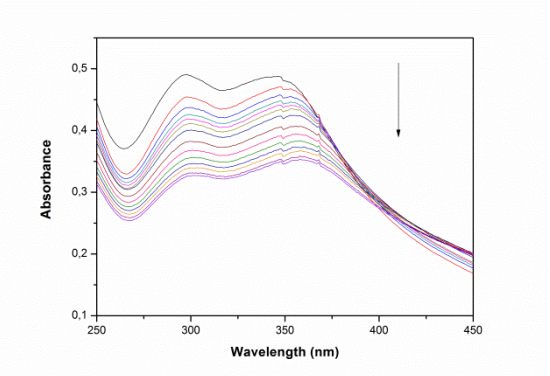


Figure 3. Spectrophotometric titration of derivative **5h** (6 μM) in 0.01 M Tris buffer (pH 7.3, 24 °C) with increasing concentration of CT DNA (from top to bottom, step 1.56μM).

Table 1. UV-Vis absorption characteristics and binding constants of compounds **5a–h.**

Compound	λ_{max1} (nm)	λ_{max2} (nm)	$\Delta\lambda_1$ (nm)	$\Delta\lambda_2$ (nm)	Hypochromism (%)		K_{sv} (M^{-1})
5a	298	-	4	-	34.78	-	0.389×10^4
5b	297	-	2	-	19.05	-	0.429×10^4
5c	300	-	2	-	21.43	-	0.481×10^4
5d	298	-	5	-	28.75	-	0.384×10^4
5e	294	-	6	-	19.57	-	0.126×10^4
5f	298	-	3	-	27.08	-	1.131×10^4
5g	297	-	2	-	13.95	-	0.332×10^4
5h	298	346	5	12	29.79	27.08	1.394×10^4

The results are similar to those previously reported by our group for comparable intercalators, methyl 2-[2-(acridin-9-yl)imino-3-R-4-oxo-1,3-thiazolidin-5-ylidene]acetates (R = *sec*-Bu, *tert*-Bu, 4-Br-C_6H_4) which were prepared from thioureas and dimethyl acetylenedicarboxylate.[14]

For better information about the DNA binding properties of the investigated spiro-acridines **5a–h**, competitive binding experiments based on the displacement of ethidium bromide (EB) from CT DNA were performed. In this assay, molecules that are able to bind to DNA *via* the same mode as EB are able to displace EB from the DNA helix. The amount of fluorescence quenching of the DNA–EB system can be used to determine the extent of intercalation between the examined molecule and DNA.[15,16] The representative emission spectra of EB bound to DNA in the absence and presence of compound **5h** is shown in Figure 4; similar reduction of the fluorescence maxima was observed for derivatives **5a–g**. Upon addition of the spiro-acridines, a significant decrease in the fluorescence intensity was observed, suggesting that the compounds bind to DNA by the intercalative mode. The fluorescence quenching of DNA-bound EB can be well described by the linear Stern-Volmer equation[17] in which the spiro-acridine derivatives are the quenchers. The Stern-Volmer quenching constants (K_{SV}) obtained from the linear quenching plot was estimated to range from 0.126×10^4 to 1.394×10^4 M^{-1} (Table 1).

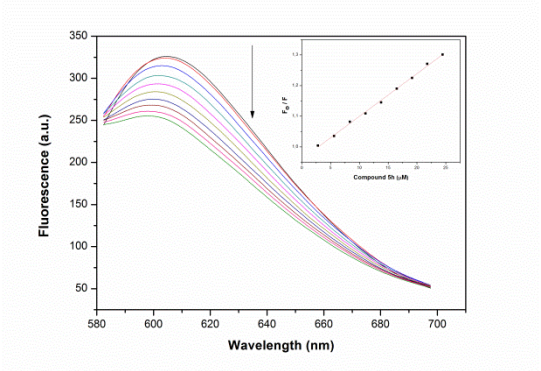


Figure 4. Fluorescence emission spectra of EB bound to CT DNA in the absence and presence of increasing amounts of derivative **5h.** Inset: the corresponding Stern–Volmer plot for the quenching process of EB by **5h** (0-25.2 μM), at 2.8 μM intervals. Arrow indicates change upon increasing concentration.

CD experiments were performed in order to define the orientation of the compounds with respect to the DNA helix. The B-form conformation of DNA shows two conservative CD bands in the UV region, a positive band at 278 nm due to the base stacking and a negative band at 246 nm due to the polynucleotide helicity.[1,15] Upon incubation of the compounds with CT DNA, the CD spectra displayed minor changes of both the positive and negative bands (Fig. 5). The helical band at 246 nm, corresponding to the extent of DNA unwinding, exhibited a decrease and slight blue shift for all compounds in the same order as that of bis-3,6-alkylamidoacridines.[18] CD spectral measurements confirmed the intercalating binding mode of the studied compounds.

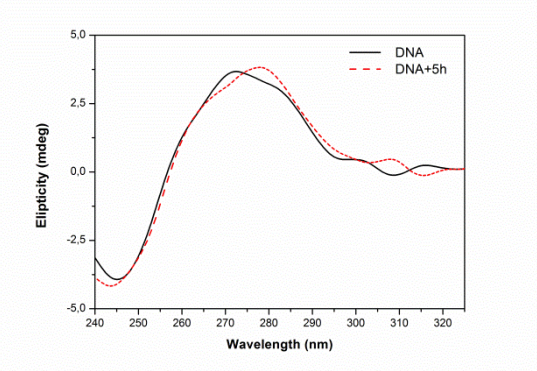


Figure 5. CD spectra of CT DNA in the absence (black solid line) and presence (red dashed line) of derivative **5h** (0.15 mM), in 0.01 M Tris buffer (pH 7.4, 24 °C).

The planar polycyclic structure of the acridines allows them to easily intercalate into double-stranded DNA, which can interfere with DNA regulatory enzymes such as topoisomerases.[19] Topoisomerases (TOPO I, II) are enzymes involved in the supercoiling of DNA, and play important roles in many aspects of DNA processing. As a result, these molecular targets are being studied for the development of a new generation of inhibitory agents.[20] There are two mechanisms by which an intercalator can affect topoisomerase activity and thereby stop cell proliferation. Firstly through intercalation, in which the binding site of the topoisomerase is occupied and formation of the enzyme–DNA complex is hindered, and secondly through the formation of a ternary complex between DNA, the intercalator and topoisomerase, a structure which is significantly more stable than the DNA–topoisomerase complex.[1,21]

A standard plasmid cleavage assay was used to study the effect of compounds **5a–h** (10, 20 and 30 μM) on calf thymus topoisomerase I by DNA gel electrophoresis. This method provides a direct means of determining whether the compounds affect the unwinding of a supercoiled (SC) duplex DNA substrate; the enzyme converts SC DNA to relaxed (R) DNA and nicked open circular (NC) DNA. If Topo I retains its normal activity, one would expect to observe the disappearance of the SC DNA band (fastest band) and the appearance of bands for R DNA and NC DNA.[22] On the contrary, if the activity of the enzyme is inhibited, no R DNA or NC DNA would be detected.

As shown in Figure 6, compounds **5a, f–h** at 30 μM inhibit TOPO I, as indicated by the negative SC form as the main band (lanes 5, 20, 23, 26), while the bands of NC and R form disappeared. However, compounds **5b–e** showed only partial inhibition of topoisomerase I at the same concentration.

Figure 6. The inhibition of human Topo I by compounds **5a–h**. Lane 1: pUC19 DNA; lane 2: DNA+ Topo I; lanes 3-5: DNA + Topo I + **5a** (10, 20, 30 μM); lanes 6-8: DNA + Topo I + **5b** (10, 20, 30 μM); lanes 9-11: DNA + Topo I + **5c** (10, 20, 30 μM); lanes 12-14: DNA + Topo I + **5d** (10, 20, 30 μM); lanes 15-17: DNA + Topo I + **5e** (10, 20, 30 μM); lanes 18-20: DNA + Topo I + **5f** (10, 20, 30 μM); lanes 21-23: DNA + Topo I + **5g** (10, 20, 30 μM); lanes 24-26: DNA + Topo I + **5h** (10, 20, 30 μM);. Topo I and pUC19 were incubated for 30 minutes at 37 °C in the presence or absence of compounds **5a–h**. SC - supercoiled DNA, R - relaxed DNA, NC - nicked open circular DNA.

In summary, we have synthesized and characterized eight novel spiro-acridines *via* the regioselective 1,3-dipolar cycloaddition reaction of an acridine dipolarophile containing an exocyclic double bond and nitrile oxide derivatives. The single crystal X-ray structure of derivative **5a** was determined. Moreover, the DNA-binding properties of these compounds were investigated by electronic absorption, fluorescence and CD spectroscopy. The experimental results showed that compounds **5a–h** bind with DNA *via* intercalation. The highest values of the K_{SV} constant were established for derivatives **5f, h** which containing electron acceptor substituents (NO_2, Br) at the *para* position (R^3) of the aromatic ring. This was in contrast to derivative **5e** with an electron donating substituent (OCH_3) at the *para* position. Derivatives **5a–d**, **g** did not significantly differ in their K_{SV} values. Compounds **5a**, **5f**–**h** showed inhibition activity against topoisomerase I, suggesting that these spiro-acridine derivatives may act as potential anticancer agents.

Acknowledgements

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Supplementary data

CCDC 1497242 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk).

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Highlights:

New spiro acridin-isoxazolines were synthesized by 1,3-dipolar cycloaddition

Single crystal X-Ray structure of spiro-acridine derivative **5a** was solved

Evaluation of DNA-binding properties by spectroscopic methods

Topoisomerase I inhibition assay