Synthesis and study of 4-hydroxymethyl-3-(alkylamino)acridines as models of a new class of DNA-intercalating–alkylating agents

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The synthesis, and the reactions with nucleophiles, of 4-hydroxymethyl-3-(dimethylamino)- and -3-(methylamino)-acridines are presented. The reactivity of both compounds in methanol and propan-2-ol is studied. The corresponding 4-methoxy- and 4-isopropoxymethyl-3-(alkylamino)acridines are obtained quantitatively. Kinetics data indicate that protonation of the acridine ring nitrogen greatly increases reaction rates, and results are in favour of a very efficient intramolecular acid–base catalysis generating quinone-imine-methide intermediates. Transetherification reactions (*i.e.*, transformation of methyl ethers into isopropyl ethers) are also observed. The reactivity with DNA is studied. Covalent binding to calf-thymus DNA is evidenced by UV–visible analysis of the modified DNA pellets. Ratios of 1 drug bound per 14 base pairs for the 3-methylamino analogue and 1 drug per 16 base pairs for the dimethyl analogue are calculated, and correspond to 50% of the drugs bound to the macromolecule.

Introduction

Alkylating agents and intercalating drugs constitute two important classes of anticancer agents. A few molecules possess the two properties, intercalation between DNA base pairs and covalent binding to the macromolecule. Natural compounds such as anthracycline antibiotics, adriamycin and daunomycin are among the most widely used drugs for cancer chemotherapy and behave as intercalating–alkylating agents. They strongly intercalate DNA and form drug–DNA covalent adducts. Their mechanism of action is still debated.[1] Another class of natural intercalating alkylating drugs is the pluramycin family.[2] Pluramycin antitumour antibiotics consist of a planar anthrapyrantrione chromophore to which is attached a side chain containing an epoxide. The family includes altromycin and hedamycin. Following intercalation of the anthrapyrantrione chromophore into DNA, the epoxide side chain is located in the major groove, allowing selective alkylation of N-7 guanine. The structurally related psorospermin xanthone antibiotic displays a similar reactivity.[3] Various synthetic intercalating alkylating agents have been designed in the past 10 years.[4] Denny and his group have extensively developed this approach by introducing various alkylating moieties to intercalating heterocycles in an effort to increase both the affinity and the selectivity for DNA.[5] They used mainly acridine as intercalator, the alkylating function (nitrogen mustards or nitrosoureas) being located on a side chain. More recently, the same group reported the synthesis and study of a hybrid molecule consisting of 9-aminoacridine linked to 3-chloromethyl-6-hydroxyindoline (*seco*-CI),[6] as precursor of the alkylating function present in the well-known family of duocarmycin antitumour antibiotics. All these natural or synthetic intercalating–alkylating agents interact by positioning the alkylating group in one groove, allowing the formation of drug–DNA adducts.[7]

In a search for new anticancer agents, we have prepared a series of heterocyclic molecules derived from 3,6-diaminoacridine.[8,9] The design of these molecules was based upon previous observations showing selective H/D exchange of the H-4 and H-5 hydrogens of this well-known intercalator. To modulate the intercalation properties of the drugs, new substituents and/or a new ring (10-aminobenzo[*b*][1,7]-phenanthroline derivatives[10]) were introduced on one side of the acridine ring. Meanwhile the reactivity of the C-4 site permitted introduction of an alkylating function, *i.e.*, a reactive CH_2X group. We anticipated that such molecules would intercalate in DNA and generate an electrophilic quinone-imine-methide intermediate directly inside the intercalation complex, thus favouring the reaction with nucleophilic sites of nucleic bases (Fig. 1). These compounds would therefore constitute a new type of intercalating–alkylating agent as the electrophilic species would be generated at the intercalation site, *i.e.*, in a 'sandwich' position between base pairs, and not in a groove as observed for the other intercalating–alkylating drugs. Along

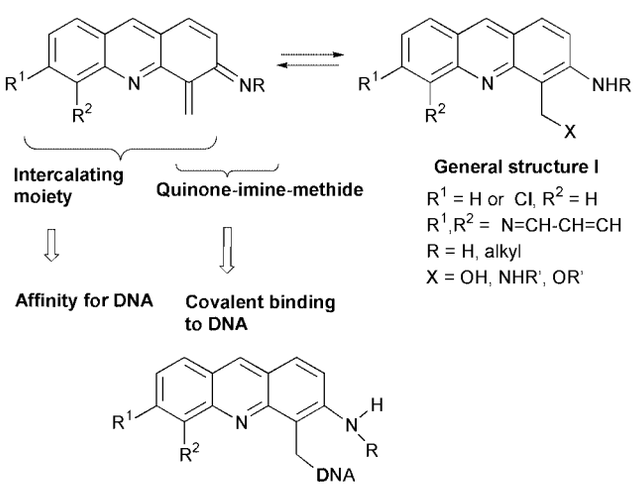

Fig. 1

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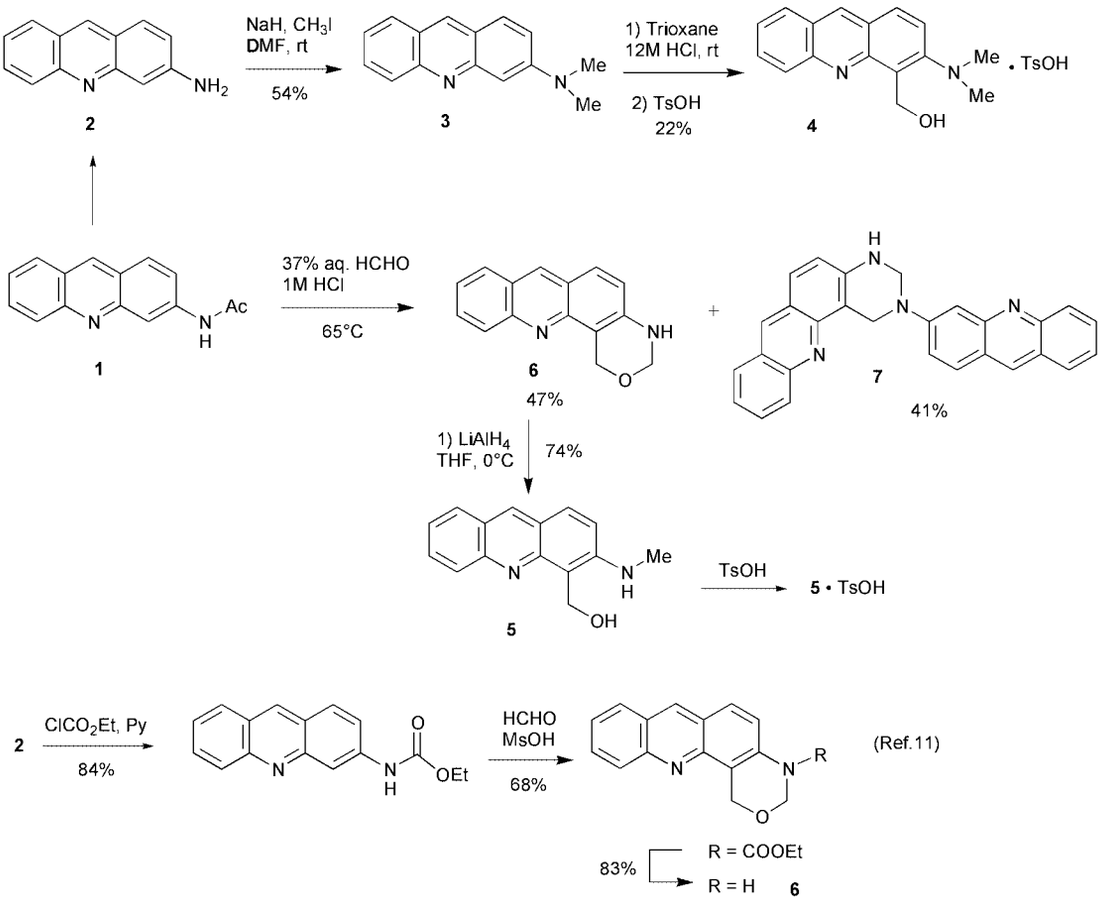


Scheme 1

these lines, large series of molecules possessing the general structure shown in Fig. 1 were prepared. For the benzo[*b*]-[1,7]phenanthroline derivatives, pharmacological data indicated that the highest cytotoxicity was found with molecules substituted at position 11, in particular with a hydroxymethyl group, or with the dihydrooxazine derivatives.[8] Similar trends were obtained with the proflavine (3,6-diaminoacridine) and 3-aminoacridine analogues. Furthermore, it turned out that some of these molecules, tested *in vivo*, displayed positive activities of tumour regression and/or increased survival time ratio,[8] the molecules containing the hydroxymethyl group in an *ortho* position to the amino group showing the highest activities.

In order to probe the validity of the intercalation–alkylation hypothesis for this family of molecules, we examined more closely the two molecules 3-dimethylamino- and 3-methylamino-4-hydroxymethylacridine **4** and **5**, possessing a benzylic-type hydroxy group in a position *ortho* to the amino substituent (Scheme 1). These molecules can be considered as the simplest models for the molecules of the family. We report here on synthesis of **4** and **5**. We show that the two drugs react with simple nucleophiles in reactions in which the primary hydroxy group is readily substituted. Solvolytic kinetic data measured for the acridine free bases and for the corresponding acridinium salts indicate an acid–base intramolecular catalysed process, that accounts for the reactivity of the drugs at physiological pH, and is compatible with transient quinone-imine-methide intermediary. DNA-alkylation properties were evidenced. A high level of covalent binding to calf-thymus DNA close to 50% of added drug was determined.

Results and discussion

Synthesis

We have previously reported on the regioselective electrophilic substitution of 3-aminoacridine or 3,6-diaminoacridine (proflavine) at a positions *ortho* to the amino groups.[9,11] We took advantage of this reactivity to prepare compounds **4** and **5**. The starting compound, 3-(acetylamino)acridine **1**, was prepared from commercially available proflavine in two steps following Martin's procedure.[12]

3-Dimethylamino-4-(hydroxymethyl)acridine **4** was obtained in three steps from **1**. Hydrolysis of the acetyl group of **1** gave 3-aminoacridine[12] **2**, which was methylated with methyl iodide in the presence of sodium hydride to give 3-(dimethylamino)-acridine **3** (54% yield). Electrophilic substitution of **3** with 1,3,5-trioxane in 12 M hydrochloric acid afforded **4** that was isolated as the toluene-*p*-sulfonate salt **4·TsOH** (22% yield).

4-Hydroxymethyl-3-(methylamino)acridine **5** was prepared by reductive ring opening of the key intermediate 3,4-dihydro-1*H*-[1,3]oxazino[4,5-*c*]acridine **6**. We have previously described the synthesis of compound **6**.[11] The methodology involved protection of the exocyclic amine of 3-aminoacridine **2** as the ethyl carbamate, followed by electrophilic substitution with formaldehyde in methanesulfonic acid yielding the ethoxycarbonyl-protected dihydrooxazine, which was hydrolysed in basic conditions to give **6** in 47% global yield. A shorter route to **6** was designed by studying both the effect of the nature of the exocyclic amino protecting group and the conditions of the electrophilic substitution. It was found that reaction of 3-(acetylamino)acridine **1** with formaldehyde in 1 M hydrochloric acid gives in one step the desired dihydrooxazine **6**, isolated in 47% yield, along with the tetrahydroquinazoline **7** (41% yield). Despite the moderate yield, this new pathway is attractive as it affords **6** in only three steps from commercially available proflavine. This route was therefore preferred to the method that we previously reported.[11] Reaction of the dihydrooxazine **6** with $LiAlH_4$ achieved ring cleavage and afforded the desired alcohol **5** in 74% yield.

The toluene-*p*-sulfonate salts, **4·TsOH** and **5·TsOH**, were

Table 1 Solvolytic data

Experiment	Compound	R^1	R^2	Product	R^1	R^2	Solvent	$t_{1/2}$	$k(\text{min}^{-1}) \times 10^4$
1	**4**	Me	H	**8**	Me	Me	MeOH	66 h	1.7
2	**4·TsOH**	Me	H	**8**	Me	Me	MeOH	31 min	220
3	**5**	H	H	**10**	H	Me	MeOH	44 h	2.6
4	**5·TsOH**	H	H	**10**	H	Me	MeOH	108 min	60
5	**4**	Me	H	**9**	Me	Pr^i	Pr^iOH	[a]	($k \ll 0.4$)
6	**4·TsOH**	Me	H	**9**	Me	Pr^i	Pr^iOH	35 min	200
7	**5**	H	H	**11**	H	Pr^i	Pr^iOH	122 h	0.9
8	**5·TsOH**	H	H	**11**	H	Pr^i	Pr^iOH	70 min	98
9	**8·TsOH**	Me	Me	**9**	Me	Pr^i	Pr^iOH	78 min	89
10	**10·TsOH**	H	Me	**11**	H	Pr^i	Pr^iOH	407 min	17

[a] No apparent reaction after 10 days. Reactions were performed in the dark at 40 °C. Solutions of 2 mg of drug in 6 cm^3 of alcohol were used (final concentrations: 1.4 and 1.3 × 10^{-3} M for the free bases of **5** and **4**, respectively, and 8.1 and 7.8 × 10^{-4} M for **5·TsOH** and **4·TsOH**, respectively). Reactions were followed by HPLC (detection at 254 and 365 nm). Pseudo-first-order kinetics were observed.

prepared by treatment of the free bases with one equivalent of toluene-*p*-sulfonic acid in ethyl acetate.

Chemical reactivity

We first determined the basicity of molecules **4** and **5**, and studied their reactivity, both as free bases and acridinium salts under solvolytic model conditions.

pK_a Determination

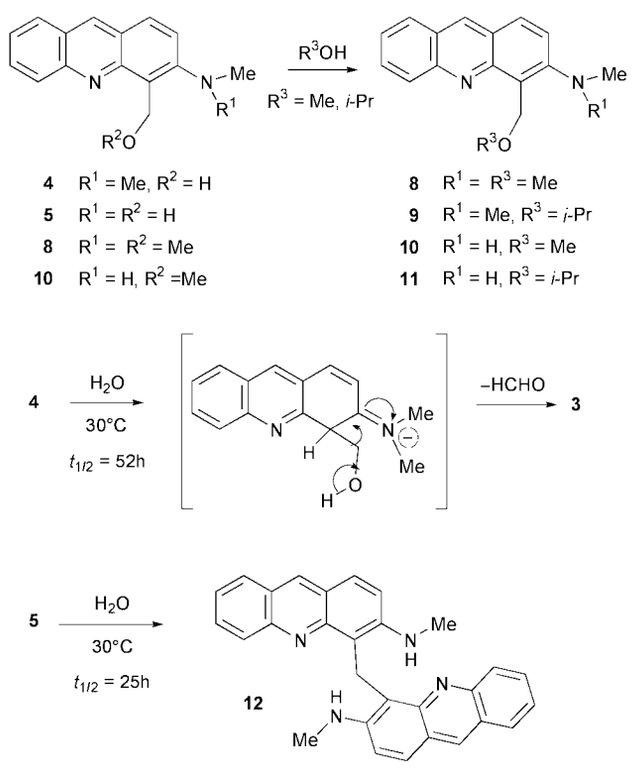
Determination of pK_as was performed by pHmetric titration in water, and values of 7.23 and 7.90 were obtained for **4** and **5**, respectively. The variations of the UV–visible spectra of the two molecules in methanol in the presence of increasing concentrations of TsOH were also examined (data not shown). Protonation occurred on the heterocyclic nitrogen as indicated by the strong bathochromic shifts observed under acidic conditions.[13] The relative geometry of the two molecules may explain the observed differences in the pK_a-values. Molecular modelling (data not shown) revealed that the two methyl groups present on the exocyclic amine in **4** are slightly twisted out from the plane of the acridine nucleus, and, on the other hand, the presence of only one methyl group allows **5** to be fully planar. Delocalisation of the amino free electron pair would therefore be more efficient in **5** than in **4**, as confirmed by the lower pK_a measured for **4**.

Solvolyses in methanol and propan-2-ol

To probe the reactivity of alcohols **4** and **5**, we studied the solvolytic behaviour of these two molecules as free bases and as toluene-*p*-sulfonate salts in methanol and propan-2-ol at 40 °C (Scheme 2).

Compounds **4** and **5**, reacting as the free bases or as the toluene-*p*-sulfonates, gave the corresponding ethers **8**–**11** quantitatively as shown by HPLC analysis. The reaction products were isolated and identified by the usual physical techniques (NMR, mass spectrometry). Kinetics of disappearance of the starting alcohols were monitored by HPLC analysis. All reactions followed pseudo-first-order kinetics and rates were calculated. The results are collected in Table 1. Different points emerge from this study. The most significant one is that, in all cases, rates are higher for the toluene-*p*-sulfonate salts than for the free bases with rate accelerations ($k_{salt}/k_{free\ base}$) ranging from 23 for **5** reacting in methanol (compare experiments 3 and 4) to more than 500 for **4** in propan-2-ol (compare 5 and 6). The slight differences observed in methanol and propan-2-ol indicate that there was no major solvent effect. Substitution of the exocyclic amino group (methylamino or dimethylamino) had also only little influence on the rates.

Solvolyses of the toluene-*p*-sulfonate salts of the methyl ethers were also studied. Compounds **8·TsOH** and **10·TsOH** reacted in propan-2-ol to give the corresponding isopropyl



Scheme 2

ethers **9·TsOH** and **11·TsOH**, respectively, in quantitative yields. The reactions followed pseudo-first-order kinetics. The rate was slightly lower for the methylamino derivative **10·TsOH** than for the dimethyl analogue **8·TsOH**. It is worth noting that although lower, the rates of formation of the isopropyl ethers from the methyl ethers are of the same order of magnitude as the rates observed for the solvolysis of the alcohols into the isopropyl ethers.

Formation of the ethers from the alcohols **4** and **5** is clear evidence of the high reactivity of the two molecules. The rationale for these observations is that assistance to ionisation by the free-electron pair of the exocyclic nitrogen generates a quinone-imine-methide intermediate, which is trapped by the solvent, methanol or propan-2-ol, to give the corresponding ether. The higher reactivity of the acridinium salts **5·TsOH** and **4·TsOH** may be explained by an intramolecular acid–base catalysis. The effectiveness of this intramolecular catalysis is emphasised by the rates of the transetherification reactions. Additional evidence for the intramolecular acid catalysis was obtained from careful analysis of the effect of acid concentration on the rates of methanolysis of **4** and **5**. Rates were measured in methanol in the presence of increased concentrations of toluene-*p*-sulfonic acid (ranging from 0 to 10 equiv-

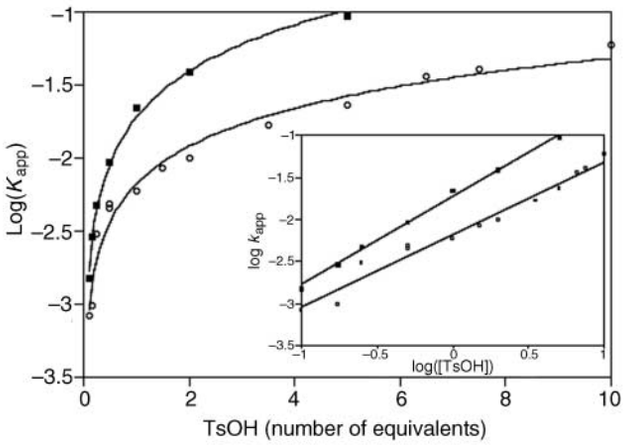


Fig. 2 Rates of formation of the ethers **8** and **10** by toluene-*p*-sulfonic acid (TsOH)-catalysed reaction of the alcohols **4** (■) and **5** (○) with alcohols R^3OH (see Scheme 2).

alents of acid relative to the drug concentration). As shown in Fig. 2, rate constants were proportional to the acid concentration expressed in equivalents of acid present in solution. Logarithmic fits were linear for the two compounds with slope-values close to 1 (insert). This Log(TsOH concentration) − Log(k_{app}) profile is a strong indication for specific acid catalysis. In the presence of acid, there is a rapid equilibrium between the acridine and its conjugate acid, *i.e.*, acridinium. An intramolecular proton transfer between the heterocyclic nitrogen and the hydroxylic substituent may then occur and thus accounts for the effectiveness of the acid catalysis (rate accelerations between reactions in the absence of TsOH and in the presence of 1 equivalent of acid, are 129 and 23, respectively, for **4** and **5**).

Reactivity in water

The reaction mechanism, as proposed for solvolysis of alcohols **4** and **5** and their salts, would regenerate the alcohols when the reactions are conducted in water. Thus, prior to studying the reactivity of alcohols **4** and **5** with DNA in aqueous solutions, we examined their behaviour in water at 30 °C and in the dark to avoid possible photochemical degradation. The toluene-*p*-sulfonate salts **4·TsOH** and **5·TsOH** were used due to the poor solubility of the free bases in water. Under these conditions, the two products exhibited slow decomposition with half-lives estimated to 52 and 25 h, respectively. These data must be compared with the kinetics of methanolysis that are, respectively, 30 min and 108 min.

The reaction products were different for the two alcohols **4·TsOH** and **5·TsOH** (Scheme 2). In water, the methylamino derivative **5·TsOH** decomposed to form a red insoluble compound that precipitated in the medium and was identified as the methylenediacridine **12**. Formation of **12** may be explained by transient generation of a quinone-imine-methide intermediate. Similar decomposition was observed previously with the oxo analogue precursor of the quinone-methide intermediate.[14] This dimerisation-like reaction was not observed in the case of the *N*,*N*-dimethylamino analogue **4**. Compound **4·TsOH** slowly transformed into 3-(dimethylamino)acridine **3** as the sole product. Protonation at position 4 with subsequent elimination of formaldehyde may account for the formation of **3** (Scheme 2). The steric hindrance introduced by the presence of the two methyl groups on the exocyclic amine may explain this difference in reactivity with **5**.

The results obtained in water as compared with the previous solvolysis data in alcohols can be interpreted by formation of the same quinone-imine-methide intermediate. In methanol, the intermediates are immediately trapped to give the corresponding ethers. In water the quinone-imine-methides are formed with kinetics that are probably of the same order as in methanol; however, the intermediates are trapped by water to regenerate the alcohols. Slow reactions can then compete, such as dimerisation favoured by aggregate formation in water in the case of **5**, or dehydroalkylation as observed for **4**.

Interaction with DNA

As with most acridine derivatives, the two molecules interact with DNA by intercalation between base pairs. In particular, negative signals characteristic of intercalated chromophores were observed in the Linear Electric Dichroism spectra of the two molecules in the presence of calf thymus DNA (data not shown).

The reactivity of compounds **4·TsOH** and **5·TsOH** with DNA was studied in water at 30 °C. Aqueous solutions of the two alcohols were added to a solution of Calf-Thymus DNA (CT-DNA) and the relative concentrations were adjusted to reach a drug-to-DNA ratio of 1 drug per 7 base pairs. The UV–visible absorptions of the drugs in the presence of DNA were similar to the absorptions of the protonated species indicating that the reactive species in the presence of DNA were the acridinium salts. To monitor the progress of the reactions, aliquots were subjected to HPLC analysis at different time intervals and the disappearance of the starting alcohols was quantified.[15] The reactions follow pseudo-first-order kinetics (k = 3.5 × 10^{-4} min^{-1} and 3.0 × 10^{-4} min^{-1}, respectively, for **4·TsOH** and **5·TsOH**). After 6 days, more than 80% of the drugs had reacted, and reactions were stopped. After extraction of the non-covalently bound drugs, DNA was precipitated and, after centrifugation, red pellets were obtained. The presence of covalently bound drug in DNA was assessed by UV–visible spectroscopy. The modified DNAs were solubilised in water and their spectra were recorded. The characteristic absorption of the acridine chromophores was observed in the visible part of the spectra (absorption at 418 nm for **4·TsOH** and 487 nm for **5·TsOH**). Quantitative analysis of the absorptions in the acridine and in the nucleic base regions revealed a ratio of 1 drug bound for 16 base pairs in the case of **4·TsOH** and 1 drug for 14 base pairs for **5·TsOH**. The (dimethylamino)acridine **3** and dimer **12** were formed as by-products respectively from **4·TsOH** and **5·TsOH**. These results clearly indicate that the two molecules bind covalently to DNA.

Pharmacological properties

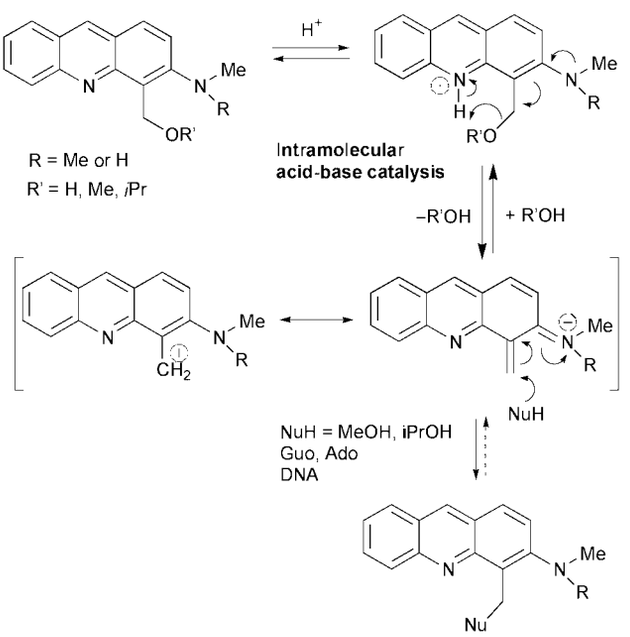
The cytotoxicity of alcohols **5** and **4**, methyl ether **8**, and dihydrooxazine **6** was tested on L1210 cancer cells. The two compounds **5** and **6** showed significant cytotoxicities (IC_{50} 0.13 and 0.20 μM, respectively). Compound **4** bearing a disubstituted exocyclic nitrogen displayed a lower cytotoxicity with IC_{50} 5.40 μM, and dimethylamino methyl ether **8** displayed the lowest cytotoxicity with IC_{50} > 10 μM.

Conclusions

The two new acridine derivatives **4** and **5** functionalised with an *o*-aminobenzylic alcohol were prepared as models to probe the DNA-alkylating potential of the molecules possessing general structure **I**. Dimethylamino-substituted alcohol **4** was prepared by electrophilic substitution of 3-(dimethylamino)acridine with formaldehyde. The key step in the synthesis of the methylamino analogue **5** was the reductive ring opening of the corresponding dihydrooxazine, itself obtained by electrophilic aromatic substitution of 3-(acetylamino)acridine with formaldehyde. The reactivity of the two molecules was studied to obtain evidence of their electrophilic potency. The two compounds reacted with methanol and propan-2-ol to give the corresponding methyl and isopropyl ethers. Comparison of the solvolytic behaviour of the free bases and of their toluene-*p*-sulfonate salts clearly demonstrates the importance of protonation of the acridine ring on the reaction rates. From these results, we propose

that an intramolecular acid–base catalysis involving the acridinium proton is implicated in the reaction. Interestingly the methyl and isopropyl ethers undergo the same catalysis in transetherification reactions. The observed reactivity may be explained by the formation of quinone-imine-methide intermediates that react with the solvent (methanol or propan-2-ol) to give the corresponding ethers. Reaction with water in the absence of added nucleophile regenerates the starting alcohols **4** and **5**. The geometry of the acridinium derivatives **4** and **5**, with the protonated heterocyclic nitrogen in a position *peri* to the reactive hydroxymethyl group, efficiently catalyses the elimination of the OH group, and allows the generation of the electrophilic species, quinone-imine-methide, in mild conditions at room temperature. Although Lau[16] reported the Lewis acid-catalysed generation of quinone-imine-methide from *o*-aminobenzyl alcohol, it should be noted that these highly reactive intermediates are generally formed by pyrolysis or photolysis of *o*-aminobenzyl alcohol derivatives.[17]

Covalent binding of the two drugs with calf-thymus DNA was evidenced. For molecules **4** and **5**, a high level of binding was found, corresponding to 1 adduct for 14 or 16 base-pairs, indicating that almost 50% of the drug was covalently bound. We propose that both **4** and **5** being at least partly protonated in water at physiological pH (7.3), form a non-covalent complex with DNA and then slowly react as electrophiles to covalently bind to DNA. Reactions are slow compared with the kinetics observed for solvolyses of the drugs in methanol and propan-2-ol. This may be due to trapping of the quinone-imine-methide intermediates by water that regenerates the starting alcohols when the geometry of the complex does not allow reaction with the bases (Scheme 3). It should be noted that in the usual cases,



Scheme 3

with most alkylating agents (halides, epoxides, nitrosoureas, *etc.*), the reaction with water competes with base alkylation, leading to deactivation of the drugs and a low ratio of covalent binding. In the present case, the reaction with water regenerates the alkylating drug thus leading to a high level of covalent binding to DNA.

All these reactivity data point to the alkylation potency of molecules **4** and **5** considered as simple models for the family of drugs possessing the general structure **I** and exhibiting high cytotoxicity.[8,9] These results favour the intercalation–alkylation hypothesis for a probable mode of action of these series of molecules at the molecular level. This reaction mechanism accounts for the data obtained with the alcohols; comparable reactivity may intervene with all analogues in which the hydroxy group is substituted for an ether, an amide, a carbamate.

Experimental

Melting points were determined using a Reicher Thermovar apparatus and are uncorrected. Except when otherwise mentioned NMR spectra were recorded at 200 MHz on a Bruker AC 200 spectrometer using solvent as the internal reference (DMSO-d_6 at δ 2.49, $CDCl_3$ at δ 7.24); the chemical shifts are reported in δ-units. The high-resolution mass spectra of pure products were recorded on Varian Mat 311 and AET MS 30 instruments and were obtained from 'Centre Regional de Mesures Physiques de l'Ouest,' Université de Rennes. Other mass spectral data were obtained from CERMAV/CNRS-Grenoble. They were recorded on a Nermag R10-10 quadripolar mass spectrometer. Absorption spectra were obtained on a Perkin-Elmer Lambda UV–visible spectrometer. Reversed-phase HPLC was performed with a μ-bondapak C18 analytical column (Waters Associates). A Waters chromatographic system was used, with two M-510 pumps and a photodiode array detector Waters 996 using Millenium 32 software. A linear gradient from 0 to 100% methanol in water, pH 2.5 (phosphoric acid), 2 cm^3 min^{-1} flow rate, was used.

3-(Dimethylamino)acridine 3

Sodium hydride (60% in oil; 1.2 g, 30 mmol) was added to a solution of 3-aminoacridine[12] **2** (1.94 g, 10 mmol) in dimethylformamide (20 cm^3) cooled at 0 °C. After 15 min of stirring, a solution of methyl iodide (2.5 cm^3, 40 mmol) in dimethylformamide (2.5 cm^3) was added dropwise at 0 °C. After 1 h of stirring at room temperature, the mixture was poured into ice–water (100 cm^3) and extracted twice with chloroform (2 × 20 cm^3). The extract was then washed with water, dried on sodium sulfate, and concentrated *in vacuo*. Compound **3** was purified by column chromatography on silica gel (100 g, elution with chloroform–methanol–ammonium hydroxide 98 : 1.75 : 0.25%) and was obtained in 54% yield (1.2 g, 5.4 mmol), mp 184–186 °C (lit.,[18] 216 °C).

3-(Dimethylamino)-4-hydroxymethylacridinium toluene-*p*-sulfonate 4·TsOH

A mixture of 3-(dimethylamino)acridine **3** (1.25 g, 5.6 mmol) and 1,3,5-trioxane (1.1 g, 12.2 mmol) in 12 M hydrochloric acid (11 cm^3) was stirred at room temperature for 16 h. The solution was then poured dropwise into a mixture of ice (50 g) and ammonium hydroxide (35 cm^3) and the precipitate thus formed was extracted three times with methylene dichloride. The organic layers were combined, washed successively with water and brine, dried on sodium sulfate, and evaporated to dryness. The residue was chromatographed on silica gel (elution with chloroform–ethyl acetate 92 : 8). The fractions containing **4** were combined and evaporated. The solid was dissolved in ethanol–ethyl acetate (1 : 1) in the presence of toluene-*p*-sulfonic acid. Compound **4·TsOH** was obtained as the toluene-*p*-sulfonate salt in 22% yield (0.55 g), mp 177–179 °C; $\delta_H(CDCl_3)$ 13.85 (s, 1H, NH), 8.88 (s, 1H, H-9), 8.77 (d, *J* 8.7 Hz, 1H, ArH), 7.76–8.01 (m, 5H, ArH), 7.41–7.32 (m, 2H, H-7, H-2), 7.21–7.31 (m, 2H, ArH), 6.47 (t, 1H, OH), 5.13 (d, *J* 4.7 Hz, 2H, $ArCH_2O$), 3.35 [s, 6H, $N(CH_3)_2$], 2.36 (s, 3H, $ArCH_3$); MS (FAB^+, glycerol) *m/z* 253 $(M + H)^+$ (Calc. for $C_{23}H_{24}N_2O_4S$: C, 65.07; H, 5.70; N, 6.60. Found: C, 64.91; H, 5.67; N, 6.59%).

Reaction of 3-(acetylamino)acridine with formaldehyde in 1 M hydrochloric acid: formation of 3,4-dihydro-1*H*-[1,3]oxazino-[4,5-*c*]acridine 6 and 2-(acridin-3-yl)-1,2,3,4-tetrahydro-pyrimido[4,5-*c*]acridine 7

A solution of 3-(acetylamino)acridine[12] **1** (0.5 g, 2.12 mmol)

and 37% aq. formaldehyde (5 cm^3, 66 mmol) in 1 M hydrochloric acid was stirred at 65 °C overnight. The mixture was then basified with dil. aq. ammonium hydroxide and extracted three times with ethyl acetate. The organic phase was washed with water, dried on sodium sulfate, and evaporated to dryness. Flash column chromatography on silica gel (elution with methylene dichloride–ethyl acetate 1 : 1) afforded compound **6** as a yellow powder in 47% yield (0.236 g, 1 mmol) and compound **7**[11] as secondary product (0.180 g, 0.44 mmol, 41%). The oxazine **6** had mp 180 °C; δ_H($CDCl_3$) 8.65 (s, 1H, H-7), 8.05 (dd, *J* 8.6 and 2 Hz, 1H, H-11), 7.85 (d, *J* 8.4 Hz, 1H, H-8), 7.65 (d, *J* 9.0 Hz, 1H, H-6), 7.60 (m, 1H, H-10), 7.40 (m, 1H, H-9), 6.90 (d, *J* 9.0 Hz, 1H, H-5), 5.45 (s, 2H, ArCH_2O), 4.90 (s, 2H, NCH_2O), 4.50 (s, 1H, NH); MS (FAB^+, NBA) *m*/*z* 236 (M^+) (Calc. for $C_{15}H_{12}N_2O$: C, 76.25; H, 5.12; N, 11.86. Found: C, 76.10; H, 5.07; N, 11.60%).

4-Hydroxymethyl-3-(methylamino)acridine 5

$LiAlH_4$ (0.164 g, 4,3 mmol) was added to a solution of the dihydrooxazine **6** (0.5 g, 2.12 mmol) in THF (25 cm^3) cooled at 0 °C. The solution was stirred for 4 h, and then poured into a mixture of water (60 cm^3) and ethyl acetate (50 cm^3). The insoluble part was filtered off, and the organic layer was separated. The aqueous phase was extracted twice with ethyl acetate, and the organic phases were collected, washed successively with water and brine, and dried on sodium sulfate. The solvent was evaporated off in the dark and the residue was triturated in diisopropyl ether. The yellow solid thus formed was filtered, giving **5** in 74% yield (0.374 g, 1.57 mmol), mp 155 °C; δ_H(DMSO-d_6) 8.77 (s, 1H, H-9), 7.97 (d, *J* 8.2 Hz, 1H, H-5), 7.95–7.91 (m, 2H, H-1 and H-8), 7.69 (m, 1H, H-6), 7.41–7.32 (m, 2H, H-7 and H-2), 6.22 (q, *J* 4.7 Hz, 1H, NH), 5.28 (d, *J* 4.7 Hz, 2H, ArCH_2O), 5.08 (t, *J* 4.7 Hz, 1H, OH), 2.98 (d, *J* 4.7 Hz, 3H, NCH_3); MS (FAB^+, NBA) *m*/*z* 239 $(M + 1)^+$ (Calc. for $C_{15}H_{14}N_2O{\cdot}0.1H_2O$: C, 75.04; H, 5.96; N, 11.67. Found: C, 74.96; H, 5.91; N, 11.66%).

Formation of the toluene-*p*-sulfonate salt, 5·TsOH. A stoichiometric amount of toluene-*p*-sulfonic acid solubilised in the minimum amount of ethyl acetate was added to a solution of compound **5** in ethyl acetate. Immediate precipitation of a red solid was observed. Compound **5·TsOH** was filtered off, washed with ethyl acetate, and dried.

Ether formation. General procedure

Compounds **5·TsOH** and **4·TsOH** (0.05 g) were dissolved in methanol or propan-2-ol (30 cm^3) and the reaction mixture was stirred at room temperature in the dark. The disappearance of the starting compound was monitored by HPLC. After 5 days of stirring, the mixture was evaporated to dryness and the residue was triturated in diisoproyl ether. The insoluble solid was filtered off, washed with diisopropyl ether, and dried over activated silica gel. Compounds **8**–**11** were thus isolated as the toluene-*p*-sulfonates.

3-(Dimethylamino)-4-(methoxymethyl)acridine, 8·TsOH. Mp 178–180 °C; δ_H($CDCl_3$) 13.66 (s, 1H, NH), 9.05 (s, 1H, H-9), 8.78 (d, *J* 8.3 Hz, 1H, ArH), 7.95–8.00 (m, 3H, ArH), 7.85 (d, *J* 8.3 Hz, 1H, ArH), 7.75 (m, 1H, ArH), 7.16–7.34 (m, 4H, ArH), 5.02 (d, *J* 4.7 Hz, 2H, ArCH_2O), 3.62 (s, 3H, CH_3), 3.23 [s, 6H, $N(CH_3)_2$], 2.38 (s, 3H, ArCH_3); MS (FAB^+, glycerol) *m*/*z* 267 $(M + H)^+$ (Calc. for $C_{24}H_{26}N_2O_4S$: C, 65.74; H, 5.98; N, 6.39. Found: C, 65.91; H, 5.88; N, 6.46%).

3-(Dimethylamino)-4-(isopropoxymethyl)acridine 9·TsOH. Mp 134–136 °C; δ_H($CDCl_3$) 9.95 (s, 1H, H-9), 8.75 (d, *J* 8.9 Hz, 1H, ArH), 7.81–7.92 (m, 5H, ArH), 7.44 (m, 1H, ArH), 7.31 (d, *J* 8.6 Hz, 1H, ArH), 7.09 (d, *J* 7.8 Hz, 2H, ArH), 5.20 (d, *J* 4.7 Hz, 2H, ArCH_2O), 4.32 [m, 1H, CH(CH_3)$_2$], 3.22 [s, 6H, $N(CH_3)_2$], 2.31 (s, 3H, ArCH_3), 1.20 [d, *J* 5.8 Hz, 6H, CH(CH_3)$_2$]; MS (FAB^+, glycerol) *m*/*z* 295 $(M + H)^+$ (Calc. for $C_{26}H_{30}N_2O_4S$: C, 66.93; H, 6.48; N, 6.00. Found: C, 66.44; H, 6.44; N, 5.89%).

4-Methoxymethyl-3-(methylamino)acridine 10. Mp 162–165 °C; δ_H(free base; $CDCl_3$) 8.50 (s, 1H, H-9), 8.00 (d, *J* 8.5 Hz, 1H, ArH), 7.83 (m, 2H, ArH), 7.56 (m, 1H, ArH), 7.25 (m, 1H, ArH), 7.15 (m, 1H, ArH), 5.40 (s, 2H, ArCH_2O), 3.20 (s, 3H, OCH_3), 3.00 (s, 3H, NCH_3); HRMS (FAB^+) (Calc. for $C_{16}H_{17}N_2O$: *m*/*z*, 253.1341 $(M + H)^+$. Found: *m*/*z*, 253.1343).

4-Isopropoxymethyl-3-(methylamino)acridine 11. Precipitation of this compound in propan-2-ol–diisopropyl ether mixture gave a resinous gum from which 20 mg (36% yield) could be isolated in a solid form. This compound easily hydrolyses to give alcohol **5** in the presence of traces of water, this precluded any further attempt of purification. Mp 130–135 °C; δ_H(TsOH salt; acetone-d_6) 9.22 (s, 1H, H-9), 8.55 (d, *J* 9.5 Hz, 1H, ArH), 8.12–8.21 (m, 2H, ArH), 7.93 (m, 1H, ArH), 7.74 (d, *J* 8.3 Hz, 2H, ArH), 7.48–7.63 (m, 3H, ArH), 7.11 (d, *J* 8.3 Hz, 2H, ArH), 5.23 (d, *J* 4.7 Hz, 2H, ArCH_2O), 3.90 [m, 1H, CH(CH_3)$_2$], 3.22 (d, *J* 4.9 Hz, 3H, NHCH_3), 2.29 (s, 3H, ArCH_3), 1.19 [d, *J* 6.1 Hz, 6H, CH(CH_3)$_2$]; HRMS (FAB^+) (free base) (Calc. for $C_{18}H_{21}N_2O$: *m*/*z*, 281.1654 $(M + H)^+$. Found: *m*/*z*, 281.1652).

PH-metric titration

Measurements were made at 25 °C and the solutions were prepared with deionised, distilled water. The ionic strength was fixed at 0.1 M in sodium chloride (Prolabo, puriss). A Tacussel Electroburex burette and a pH meter (Tacussel, Iono Processeur II) connected to a Hewlett Packard microcomputer were used. The electrodes (glass and saturated calomel) were calibrated with standard buffer solutions. Compounds **4** and **5** (5.0×10^{-4} M) containing 1 equivalent of perchloric acid was titrated with standardised 10^{-2} M sodium hydroxide. Analysis of the titration data was performed using the SUPERQUAD program.[19]

Solvolyses

Kinetics experiments were performed in a thermostatted bath, at 40 °C in the dark. The concentrations of the starting compounds were 1.4 and 1.3×10^{-3} M for **5** and **4**, respectively, 8.1 and 7.8×10^{-4} M for **5·TsOH** and **4·TsOH**, respectively, and 7.6 $\times 10^{-4}$ M for **8·TsOH**. Solutions were obtained by dissolving 2 mg of the tested compound in 6 cm^3 of methanol or propan-2-ol. For the solvolyses in methanol in the presence of toluene-*p*-sulfonic acid, 1.4×10^{-2} M stock solution of TsOH in methanol was prepared. Solutions containing 0.1 to 10 equivalents of TsOH (relative to **4** or **5**) were prepared by diluting the stock solution of acid in methanol and adding the tested compound **4** or **5** (1.3×10^{-3} M in drug). The disappearance of the starting material and the formation of the reaction products were monitored as a function of time by removing aliquots (5 mm^3) and injecting them onto the HPLC column. Peak heights or peak areas of UV chromatograms were used to determine pseudo-first-order rate constants. All solvolyses were followed up to 95% conversion, and duplicate experiments were always run. In all the experiments, controls established that each of the products could be quantitatively analysed in a satisfactory manner. This was achieved by independent synthesis of pure samples and calibration of the HPLC experiments.

Reaction with DNA

Stability in water. Stock solutions of compounds **4·TsOH** and **5·TsOH** were prepared by dissolving 1 mg of tested drug in

4 cm^3 of water. Milli-Q (Millipore water purification system)-grade water was used. At $t = 0$, 1 cm^3 of stock solution was diluted with water to 11.5 cm^3 to reach final drug concentrations of 5.0×10^{-5} M and 5.2×10^{-5} M for **4·TsOH** and **5·TsOH**, respectively. The reactions were performed at 30 °C in a thermostatted bath in the dark. The disappearance of the starting alcohol was monitored by HPLC. When all the starting alcohol had reacted, the reaction products were isolated. *N*-Methyl alcohol **5·TsOH** was quantitatively transformed into 4,4′-methylenedi[3-(methylamino)acridine] **12**. *N*,*N*-Dimethylamino alcohol **4·TsOH** quantitatively gave 3-(dimethylamino)-acridine **3** that was identified by comparison with an authentic sample.

4,4′-Methylene-di[3-(methylamino)acridine] ***12***. Red solid; $\delta_H(CDCl_3)$ 9.32 (s, 2H, NH), 8.51 (s, 2H, ArH), 8.20 (d, *J* 8.6 Hz, 2H, ArH), 7.88 (d, *J* 7.4 Hz, 2H, ArH), 7.76 (d, *J* 9.2 Hz, 2H, ArH), 7.75–7.68 (m, 2H, ArH), 7.39–7.32 (m, 2H, ArH), 7.16 (d, *J* 9.2 Hz, 2H, ArH), 5.32 (s, 2H, CH_2), 2.86 (s, 6H, 2 × CH_3); MS (FAB^+, NBA) *m*/*z* 429 $(M + H)^+$ (Calc. for $C_{29}H_{24}N_4 \cdot 1H_2O$: C, 78.00; H, 5.87; N, 12.55. Found: C, 77.55; H, 6.66; N, 12.06%).

Covalent binding. Stock solutions of mono- and dimethylamino alcohols **5** and **4** (2×10^{-4} M) were obtained by dissolution of 4.25 mg of **4** or 4.11 mg of **5** in 50 cm^3 of water. A stock solution of calf-thymus DNA (9.2×10^{-4} M) in water was also prepared. At $t = 0$, 2.5 cm^3 of the drug solutions were mixed with 3.8 cm^3 of DNA solution, and diluted with water to 10.5 cm^3. A drug-to-DNA ratio of 1 drug per 7 base pairs was thus obtained. Disappearance of the drug was monitored at different time intervals by HPLC. To quantify the binding to DNA, aliquots (0.75 cm^3) were taken, and extracted three times with *n*-butanol to remove the drug not covalently bound to DNA. The modified DNA was precipitated from the solution by adding 60 mm^3 of aq. sodium acetate and 1 cm^3 of ethanol. The mixture was kept for 2 h at −18 °C and then centrifuged (14 000 ***g***; 30 min; 4 °C). The DNA pellet was dissolved in 1 cm^3 of water and the same extraction–precipitation procedure was performed a second time. The DNA pellet thus obtained was dissolved in water (0.25 cm^3) and analysed by UV–visible spectroscopy. The amount of drug covalently bound to DNA was estimated by measuring drug absorption at 485 nm (ε/dm^3 mol^{-1} cm^{-1} 6150) for **5** and 360 nm (ε/dm^3 mol^{-1} cm^{-1} 6450) for **4**, and at 260 nm where both the drug and DNA absorb. The absorption at 260 nm corresponding to DNA was corrected by the absorption of the drug. For **5**, $OD_{260}/OD_{485} = 3$ and $OD_{DNA} = OD_{found} - 3 \times OD_{485}$, and for **4** $OD_{260}/OD_{360} = 2.9$ and $OD_{DNA} = OD_{found} - 2.9 \times OD_{360}$.

Kinetics experiments. The disappearance of the alcohols **5·TsOH** and **6·TsOH** in the presence or absence of DNA was followed by HPLC. The reactions were performed in the dark at 30 °C. A ratio of 1 drug per 2.5 DNA base pairs was used (DNA concentration 1.3×10^{-4} M and drug concentration 5.2×10^{-5} M). Pseudo-first-order kinetics were observed. Reactions were performed in triplicate.

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