# Potential Antitumor Agents. 39. Anilino Ring Geometry of Amsacrine and Derivatives: Relationship to DNA Binding and Antitumor Activity

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The clinical antileukemic drug amsacrine and analogues are thought to exert their biological activity by binding tightly but reversibly to DNA, with the acridine chromophore intercalated and the anilino group making additional binding contact in the minor groove of the double helix. In this binding model the steric environment around the 3'- and 5'-positions of the anilino ring is crucial. Two 3',5'-disubstituted analogues of amsacrine have been prepared, and their conformation, DNA binding properties, and antitumor activity were determined and compared with corresponding unsubstituted and 3'-substituted compounds. Addition of 3'- and 3',5'-substituents have little effect on minimum-energy conformations of the anilino side chain but have significant effects on DNA binding and biological activity. Monosubstitution lowers binding constants several-fold, but intercalative binding with extensive drug–base pair overlap is retained. Disubstitution lowers binding further, and although the binding is still intercalative as assessed by unwinding angles, it appears to occur with little drug–base pair overlap, as determined by high-field NMR studies of DNA imino proton shifts. These changes in DNA binding are accompanied by an abrupt change in biological activity, with the 3',5'-disubstituted analogues proving inactive and nontoxic even though other physicochemical properties, such as lipophilicity and stability, remain within acceptable limits. This study provides further evidence that the binding of drugs to DNA has a critical influence on their biological activity.

In recent years, DNA-intercalating[1,2] agents have been intensively studied and developed as potential antitumor agents. Intercalative binding initiates events leading to cell death by inhibition of nucleic acid synthesis and/or by the induction of strand breaks.[3] However, a great many DNA-intercalating ligands that bind equally tightly possess no antitumor activity.[4] In fact, elucidation of the structural features that distinguish active and inactive drugs of this general class, as well as an understanding of the underlying physical phenomena for this dichotomy, is a current central problem in antitumor drug development.

Recent studies[4] of a large series of DNA intercalators suggest that an important phenomenon is long residence times of a particular drug molecule *at a particular site* on the polymer. It can be envisaged how this could provide long-lived blocks to the passage of replication and/or transcription enzymes.

A majority of the tumor-active intercalating agents have attached to the chromophore a side chain bearing a cationic function. This side chain is essential for biological activity, and it is reasonable to suggest that the additional electrostatic binding of this group results in slower dissociation rates from particular sites on the DNA.[4,5]

A smaller group of tumor-active ligands (e.g., actinomycin D, the phenylphenanthridinium cations, and the 9-anilinoacridines) possesses a sterically demanding, rather than a cationic, side chain which is again essential for biological activity.

**Scheme I**
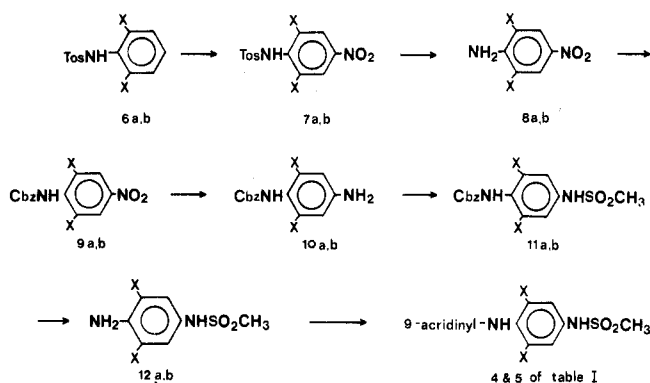


The 9-anilinoacridines form an important class of antitumor agents of this general type and include the clinical antileukemic drug amsacrine (*m*-AMSA, compound **3** in Table I).[6] The exact geometry of the binding of amsacrine and related 9-anilinoacridines to DNA has yet to be determined, but the available experimental results are consistent with the proposal that these compounds bind by intercalation of the acridine chromophore between the base pairs, with the anilino ring lodging in the minor groove.[6] In this binding mode, the 1- and 2-positions of the acridine ring are occluded by the sugar–phosphate chains of the DNA, while the 4- and 5-positions are oriented toward the major groove, free of steric restraint. The anilino 1'- and 2'-positions lie in the minor groove, also reasonably free from occlusion by the DNA in the binding model. A recent extensive QSAR study of the antileukemic activities of 9-anilinoacridine derivatives suggested that this binding

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mode is essential for biological activity,[7] by demonstrating the importance of the steric effect of substituent groups placed at different positions around the 9-anilinoacridine nucleus. While the 1′-position appeared free of steric restraint, the acceptable size of groups in the 3′-position was severely limited. A similar qualitative study of the in vitro antileukemic potency of 9-anilinoacridine derivatives showed the same pattern, confirming that the in vivo results were not due to pharmacological or stability factors.[8]

In the proposed model for the binding of 9-anilino-acridine derivatives to DNA, the steric environment around the 3′- and 5′-positions of the anilino ring is of particular interest, for this is expected to have a considerable influence on the orientation of the anilino side chain with respect to the acridine chromophore. In order to study this aspect of 9-anilinoacridine chemistry, the 3′,5′-dimethyl (4) and -dimethoxy (5) derivatives of 4-(9-acridinylamino)methanesulfonanilide (1) were prepared. Their conformational, DNA-binding, and antitumor properties were studied and compared with those of the corresponding 3′-substituted analogues (2) and amsacrine (3).

**Chemistry.** The anilino side chains for compounds 4 and 5 were elaborated by the general method of Scheme I from 2,6-dimethylaniline and resorcinol dimethyl ether, respectively. The nitration of various protected derivatives (acetylamino, formamino) of 2,6-dimethylaniline has been shown[9] to yield largely the 3-nitro isomer, due to steric inhibition of the usual mesomeric effects. However, nitration of the tosyl derivative under conditions of moderate acidity (acetic/nitric acids) was found to give exclusively the 4-nitro isomer in 80% yield,[10] and this was the method employed for both the dimethyl and dimethoxy derivatives. The structures of the nitro compounds were confirmed by NMR, which showed the expected singlet at 7.46 ppm (dimethoxy compound) from the isolated aromatic protons.

A second consequence of the steric crowding of the 1-position in these molecules is to make hydrolytic removal of the amine-protecting function exceedingly difficult after the activating 4-nitro group has been removed. Thus, the tosyl compounds (7) could be hydrolyzed in cold $H_2SO_4$ to the nitroanilines 8, but when the nitroanilines 8b was converted to 4-(trifluoromethyl)-3,5-dimethylmethanesulfonanilide, hydrolytic removal of the trifluoromethyl group to give the desired side chain (12b) could not be achieved cleanly.

Consequently, the nitroanilines 8 were converted to the benzyloxycarbonyl derivatives 9; when standard procedures were followed to obtain 11, the benzyloxycarbonyl protecting groups were removed by hydrogenolysis to provide the desired products 12.

The experimental conditions required for coupling of the amines 12 with 9-chloroacridine to provide compounds 4 and 5 of Table I were a measure of the increased degree of steric crowding around this position. The acid-catalyzed coupling of aromatic amines, such as aniline, with 9-chloroacridine proceeds readily in anhydrous solvents, such as methanol or N-methylpyrrolidone, being complete in about 10 min in N-methylpyrrolidone at 20 °C, and almost immediately in refluxing methanol. In contrast, coupling of the hindered amines 12 with 9-chloroacridine in N-methylpyrrolidone and a trace of acid did not commence
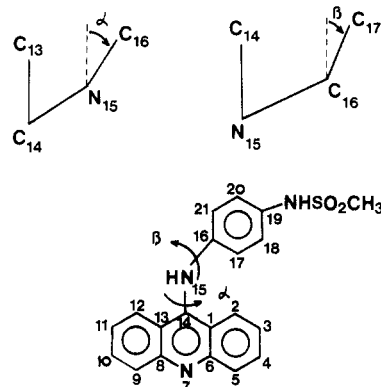
**Figure 1.** Definition of torsion angles $\alpha$ and $\beta$ for amsacrine derivatives. This is not the usual numbering of amsacrine derivatives but identifies every atom uniquely.

(as evidenced by a red coloration) until the temperature was raised to 100 °C but then proceeded smoothly to give the products 4 and 5 in good yield.

## Results and Discussion

**Conformation of the Anilino Ring.** Table I gives some physical and biological data for a series of 9-anilinoacridines having varying levels of steric crowding around the side-chain junction. The degree to which this alters side-chain geometry can be conveniently monitored by the alteration in the torsion angles $\alpha$ and $\beta$. Torsion angle $\alpha$ is defined by the four atoms C13–C14–N15–C16, and torsion angle $\beta$ is defined by the atoms C14–N15–C16–C17, as shown in Figure 1 and using the atom numbering defined there.

It might be expected that the anilino ring of AMSA derivatives would adopt a minimum-energy conformation with torsion angle $\alpha$ close to 90° and torsion angle $\beta$ close to 180° (as defined in Figure 1), in order to minimize the nonbonded interactions between H2 and H17 (or equivalently between H12 and H21). Simple rigid-geometry minimum-energy calculations, which take into account atomic electrostatic potentials but do not allow for angle deformation or torsion potentials, give for AMSA (1) a value of 81° for $\alpha$ and 145° for $\beta$, indicating that relief of the above nonbonded interactions is indeed the main driving force for adoption of this conformation. The angle of 145° for $\beta$ implies a twisting of the anilino ring out of the anilino N15–H15 plane by 35°.

This calculated structure is somewhat at variance with the determined crystal structures for AMSA (1).[11] The main difference is in torsion angle $\alpha$, which is determined as 24°. The main reason for the difference is that free rotation about the C14–N15 bond (assumed in the calculations) cannot take place, due to the considerable degree of conjugation in this bond. It has already been found that electronic effects of substituents on the anilino ring in 9-anilinoacridine derivatives are efficiently transmitted to the acridine ring, where they have the expected effects on the $pK_a$ of the acridine nitrogen.

The high degree of transmission of electronic effects from the anilino to the acridine rings is shown[12] by eq 1,

$$pK_a = -2.03 \ (\pm 0.08) \ \sigma_p + 7.27 \tag{1}$$

$$n = 17, r = 0.984, s = 0.172$$

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Table I. Physical and Biological Data for Amsacrine Analogues

| no. | $R_1$ | $R_2$ | mp, °C | formula | $Rm^a$ | $pK_a^b$ | $t_{1/2}{}^c$ | $\alpha$ | $\beta$ | AT | GC | L1210 $ID_{50}{}^f$ | OD$^g$ | ILS$^h$ max |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | H | H | | | 0.00 | 7.19 | 0.92 | 81 (24) | 145 (125) | 1.06 | 1.38 | 35 | 66 | 131 |
| 2 | CH₃ | H | | | 0.15 | 7.36 | 54.0 | | | 1.40 | 0.94 | 100 | 60 | 60 |
| 3 | OCH₃ | H | >360 | $C_{22}H_{21}N_3O_2S\cdot HCl$ | 0.18 | 7.43 | 1.8 | 81 (20) | 214 (224) | 0.79 | 1.06 | 35 | 9 | 111 |
| 4 | CH₃ | CH₃ | | | 0.41 | (7.60)$^i$ | >70 | | | <0.02 | <0.02 | >10⁴ | >500 | inact |
| 5 | OCH₃ | OCH₃ | | $C_{22}H_{21}N_3O_4S$ | 0.36 | (7.63) | >70 | 56 | 216 | <0.02 | <0.02 | 4900 | >500 | inact |

$^a$ Rm = chromatographic measure of drug lipophilicity, determined by the methods outlined in ref 25. $^b$ Acridine $pK_a$ values determined spectrophotometrically in 20% aqueous DMF (ref 26). $^c$ $t_{1/2}$ = the half-life of the drug, in minutes, in the presence of 2-mercaptoethanol under standard conditions detailed in ref 27. $^d$ Torsion angles computed as described in the text or (in parentheses) taken from the X-ray crystal data of ref 11 and 13. $^e$ Maximum shift, in parts per million of the center of mass of the AT and GC imino proton envelopes, calculated by the methods described in the text from experimental data obtained at 300 MHz as outlined in Figure 2 and ref 4. $^f$ $ID_{50}$ = the nanomolar concentration of drug that when added to cultures of murine L1210 leukemia cells for 70 h reduces the cell count by 50% compared to controls. $^g$ OD = optimal drug dose administered intraperitoneally as a solution in 0.1 mL of 30%, v/v, ethanol/water on days 1, 5, and 9 after intraperitoneal injection of 10⁶ P388 leukemia cells (ref 28 and 29). $^h$ ILS = percent increase in life span of treated animals over that of groups of control animals injected with tumor alone. Values of ILS greater than 20% are considered statistically significant. $^i$ $pK_a$ values in parentheses were calculated by subtraction of 0.25 $pK_a$ unit from values determined for the corresponding de-1'-NHSO₂CH₃ derivatives. It was not possible to determine values directly for compounds 4 and 5 due to interference from ionization of the 1'-NHSO₂CH₃ group.
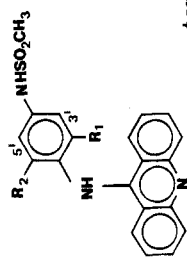
where the $\sigma_p$ values of substituents at the 1'-position are seen to be closely correlated with acridine $pK_a$. For a larger series of substituted 9-anilinoacridines, the effect of substituents placed on either the acridine or anilino rings on the acridine nitrogen $pK_a$ was similar, as shown[7] by the two negative $\rho$ values in the Hammet equation (eq 2).

$$pK_a = -2.33\ (\pm 0.14)\ \sigma_{acridine} - 1.44\ (\pm 0.14)\ \sigma_{acridine} + 7.10 \quad (2)$$

$$n = 176,\ r = 0.939,\ s = 0.259$$

Further evidence for the conjugation of the C14–N15 bond comes from the crystal structures themselves,[11,13] where a bond length of 1.35–1.36 Å is considerably shorter than usual (cf. the 1.43-Å C19–N22 bond).

Simple energy calculations for the crystal structure conformation show considerable nonbonded interactions between H-2 and H-17, which indicate that the molecule has considerable internal strain. This is evidenced in the crystal structure by the widening of the C13–C14–N15 angle to 127.2° (from an unstrained angle of 120°) and a deformation of the C14–N15 bond 8° from the acridine ring plane.[15] Thus, the conformation of the side chain results from a balance between the nonbonded interactions tending to increase torsion angle $a$ and the partially conjugated C14–N15 bond resisting this torsional twisting.

Very small effects on the degree of conjugation in the C14–N15 and N15–C16 bonds exerted by differing acridine and/or aniline substitution might thus be magnified into larger changes in minimum-energy conformations of the side chain.

Attachment of a methoxy group to position C21 (to provide the clinical drug amsacrine) results in little change in either the calculated or measured[13] values of torsion angle $\alpha$, which is dictated largely by the H2–H17 nonbonded interaction (computed value) or by the result of this interaction and the degree of conjugation of the C14–N15 bond (crystal structure value).

Torsion angle $\beta$ is calculated as 214°, placing the methoxy group as far away from the chromophore as possible and positioning C17 as far as possible away from H2. Interestingly, this conformation, with the anilino ring at an angle of 34° to the H15–N15–C19 plane, is very similar to that seen for the anilino ring in the crystal structure of amsacrine, namely, 44°.[13]

Addition of a second methoxy group to provide compound 5 has surprisingly little effect on the calculated minimum-energy conformation. Torsion angle $\beta$ is identical with that of amsacrine, and the only change is a slight closing of the $\alpha$ angle to 56°, presumably to relieve developing nonbonded interactions between H2 and the newly created methoxy group of C17. The total van der Waal energy computed for compound 5 in the minimum-energy conformation is 12.56 kcal/mol, very little more than for amsacrine (12.03 kcal/mol). Thus, introduction of the second methoxy group does not result in a great deal of extra strain from nonbonded interactions at the minimum-energy conformation. However, it may well limit the degree of flexibility of the side chain by increasing the potential barriers around that conformation. No crystal structure data are yet available for the disubstituted compounds, but a comparison of the measured $pK_a$ values of the compounds of Table I suggests little change in the

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Table II. DNA Binding Properties of
Amsacrine Analogues

| | free drug | | DNA complex | | $K^a$ | unwinding |
| no. | $\lambda_{max}$, nm | $\epsilon$ | $\lambda_{max}$, nm | $\epsilon$ | poly(dA-dT) | angle,[b] deg |
|---|---|---|---|---|---|---|
| 1 | | | 414 | 7600 | $1.5 \times 10^6$ | 20.9 |
| | 433 | 12 400 | 440 | 8200 | | |
| 2 | 412 | 14 200 | 420 | 8200 | $3.8 \times 10^5$ | 19.5 |
| | 432 | 13 400 | 440 | 8300 | | |
| 3 | | | 420 | 7700 | $2.9 \times 10^5$ | 20.5 |
| | 434 | 12 000 | 442 | 8500 | | |
| 4 | 412 | 12 400 | 418 | 9700 | $4.6 \times 10^4$ | ~20 |
| | 435 | 10 900 | 440 | 8600 | | |
| 5 | 414 | 11 200 | 418 | 9600 | $4.9 \times 10^4$ | ~19 |
| | 435 | 11 400 | 440 | 9700 | | |

[a] Binding constant to poly(dA-dT) was determined by a
fluorometric method and was corrected for quenching
(ref 28). [b] Corrected for unbound drug (see text).

actual conformation of the side chains. Addition of a
3'-OCH$_3$ group (which does not significantly alter the
side-chain conformation, as determined by the crystal
structures of 1 and 3) raised the measured p$K_a$ by 0.24 of
a unit, from 7.19 to 7.43;[11] addition of a 3'-CH$_3$ group to
give 2 raised it by 0.17 of a unit to 7.36. Further addition
of a 5'-OCH$_3$ group to give compound 5 results in a further
rise in p$K_a$ of 0.20 unit to 7.63, with a similar increase
found for addition of a second methyl group. A significant
change in side-chain conformation would be expected to
reduce the p$K_a$ due to lowering of the degree of conjugation
about the C14-N15 bond. As an example, 9-aminoacridine
and 9-(methylamino)acridine are strong bases of p$K_a$ about
9.5, but 9-(dimethylamino)acridine, where effective con-
jugation of the nitrogen with the ring is sterically pre-
vented, has a p$K_a$ (7.35) more than 2 units lower.[14]

The above evidence suggests that progressive addition
of steric bulk about the anilino ring positions adjacent to
the nitrogen does not result in any marked alteration in
the minimum-energy conformation of the side chain but
may raise the steepness of the potential barriers around
that conformation.

**DNA Binding Properties.** The DNA binding prop-
erties of AMSA (1) to calf thymus DNA have been well
characterized.[15,17] It has an association constant of 5.5 $\times$
$10^5$ M$^{-1}$ at an ionic strength of 0.01 and pH 7.0,[16] with an
unwinding angle of 20.9° fully consistent with an inter-
calative binding mode.[15] Addition of a 3'-methoxy group
to provide amsacrine (3) resulted in decreased binding.[16]
Addition of a second sterically demanding methyl or
methoxy group at the 5-position (compounds 4 and 5) lead
to a further decrease in DNA binding, with equilibrium
dialysis experiments indicating association constants for
4 and 5 to calf thymus DNA of approximately 7 $\times$ $10^3$ M$^{-1}$
at 0.01 ionic strength and pH 7.0. This low binding com-
plicates the interpretation of unwinding angles, as only 32
and 34% of compounds 4 and 5, respectively, were bound
under the conditions of the unwinding assay. Thus, the
apparent unwinding angles of 8.3° and 6.6° respectively
provide values of 26° and 19° when corrected for unbound
drug (Table II). Even allowing for possible inaccuracies
in the large corrections involved, these results suggest that
4 and 5 bind by intercalation.

Binding constants for the compounds to the copolymer
poly(dA-dT) were determined by using the ethidium dis-
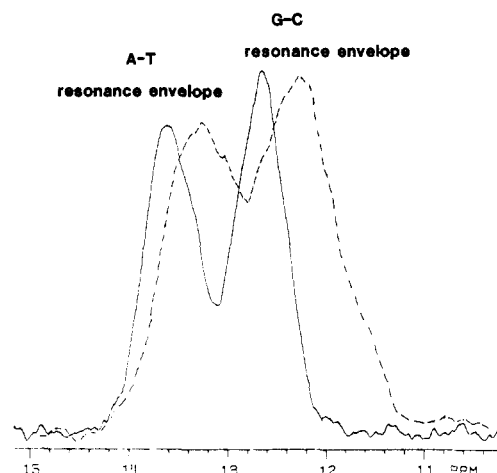placement assay[16,17] (which assumes an intercalative



**Figure 2.** Low-field portion of the $^1$H NMR spectrum at 300 MHz
of short (approximately 50 base pairs) fragments of chicken
erythrocytes DNA at 35 °C in 0.1 M NaCl, 0.01 M sodium ca-
codylate buffer. The solid line is the trace (4000 accumulated
scans) for free DNA, and the broken line is for the same DNA
sample when AMSA (1) is added at the ratio of one drug molecule
per 12.5 base pairs. See ref 4 for further details.

binding mode), and the results are given in Table II.
Again, addition of a 3'-methyl or 3'-methoxy group to 1
to give 2 and 3 lowered binding 5-fold. Addition of a
second substituent group (compounds 4 and 5) reduced
binding a further 6- to 8-fold.

The above results show a progressive decrease in binding
energy as the steric bulk around the anilino ring is in-
creased but no dramatic change in the binding mode as
determined by unwinding angle data. For compounds 1-5,
the electrostatic component of binding would be expected
to remain constant (all p$K_a$ values are above 7.0). The
relationship between DNA binding and ionic strength for
amsacrine (3)[17] is similar to that suggested by Record et
al.[18] and predicts that if electrostatic forces only are op-
erating, the binding constant would be of the order of $10^2$
M$^{-1}$. This suggests that the anilino ring provides additional
binding energy, probably by lying in the DNA minor
groove,[6] and that substitution of the anilino ring decreases
overall binding energy by inhibiting full intercalation of
the acridine chromophore and thus reducing stacking en-
ergy.

A sensitive and independent measure of drug-DNA
stacking interactions is provided by measuring the drug-
induced perturbations of the imino proton resonances of
DNA by high-field $^1$H NMR spectroscopy.[19] Under
suitable conditions, resonances from the H-bonded imino
protons of A-T and G-C base pairs can be observed in
aqueous solution (Figure 2). These protons resonate at
about 10-12 ppm, well clear of other DNA and drug res-
onances. They are observable in aqueous solution, since
they are protected from solvent exchange in double-helical
DNA by the base pairs stacked above and below them.
Thus, the observance of these resonances is also a measure
of the degree of DNA duplex formation. The position of
these resonances is determined primarily by whether the
proton is from an A-T or a G-C base pair, with the G-C
resonances occurring about 1-ppm upfield of the A-T
resonances. A secondary determinant of the exact position

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Acids Res.* **1982**, 10, 749.

of a particular resonance is the nearest neighbor and next nearest neighbors of the base pair in question, as these exert considerable ring-current shielding.[20] Under suitable conditions,[4] all the A-T and G-C resonances can be resolved from each other, resulting in two distinct envelopes, as seen in Figure 2.

When a drug is bound to the DNA by intercalation, the imino protons on the base pairs adjacent to the insertion site are separated from each other, losing their nearest-neighbor shielding. This is, however, replaced by the larger ring-current shielding of the drug chromophore, with the net effect being an upfield shift of the resonance in question. Under conditions of fast exchange, titration of DNA with an intercalating drug results in a steady upfield shift of the imino proton resonances. At the highest level of drug binding reached (1 drug molecule per 12.5 base pairs), the shifts for the A-T and G-C envelopes for AMSA (1) was, respectively, 0.17 and 0.22 ppm (Figure 2). At this ratio, assuming all the drug bound and no base-pair selectivity, the total average occupancy of either of the two immediately adjacent sites over a time period much longer than the exchange rate (i.e., the NMR time scale) will be 16%. The observed shift is thus 16% of the absolute shift exerted by the particular ligand, and by using such simple calculations, the absolute shift values for the AMSA analogues are recorded in Table I. Both AMSA (1) and the two monosubstituted compounds 2 and 3 show average absolute shift values of between 0.9 and 1.3 ppm, clearly indicative of intercalative binding. In contrast, the two disubstituted compounds 4 and 5 have shift values of essentially zero, even though it can be calculated that most of the drug will be bound under the experimental conditions. This was verified by an equilibrium dialysis experiment.

This result suggests that the addition of sufficient steric bulk to the anilino ring decreases overall DNA binding by preventing full stacking overlap with the base pairs in the DNA intercalation site, possibly because of raised potential energy barriers that limit conformational flexibility. In the resultant binding mode (of reduced stability), the acridine chromophore 4 and 5 can penetrate sufficiently far to promote significant unwinding of the DNA, without entering far enough to allow a significant shielding of the adjacent DNA imino protons.

**Biological Activities.** The data of Table I indicate a marked break in both in vivo and in vitro antitumor activity across the series. Addition of the 3'-OCH$_3$ group to 1 provides the clinical agent amsacrine (3), which is several-fold more dose potent in vivo. Addition of a 3'-CH$_3$ substituent to give 2 lowers dose potency and in vitro activity, but antitumor activity is preserved. In contrast, the 3',5'-disubstituted compounds 4 and 5 have at least 100-fold lower activity in culture and are inactive and nontoxic in vivo up to a dose of 500 mg/(kg day) (the highest feasible dose level). The in vitro activity is lower than almost all of the substituted 9-anilinoacridine derivatives previously reported,[8] even though they show great stability to thiolytic cleavage (one cause of instability in tissue culture). While the compounds are more lipophilic than the monosubstituted ones, this cannot be the reason for their inactivity, for many other highly active AMSA derivatives of higher lipophilicity exist.

## Conclusions

Several studies with different series of DNA-intercalating drugs, including anthracyclines,[21] ellipticines,[22] and

9-anilinoacridines,[23] have shown a positive correlation between antitumor activity and the ability of the compound to bind DNA, as measured by association constants. Such studies serve to reinforce the widely held view that the cytotoxicity of such compounds is mediated by their direct interaction with DNA.

However, it is clear from more detailed studies that there are at least three aspects of drug–DNA interaction that must be considered to be important for biological activity: binding mode or geometry, base-pair selectivity, and kinetics.[4] The influence of binding mode on the biological activity of a series of proflavine derivatives has recently been reported.[24] As the steric bulk of alkyl groups attached to the proflavine chromophore was increased, a change in primary binding mode from intercalative to outside binding was observed, this change coinciding with an abrupt loss of biological activity.

The present work shows that addition of steric bulk to the 3'- and 5'-positions of the anilino ring of 9-anilinoacridine derivatives has relatively small effects on the minimum-energy conformation of the side chain, as determined by simple energy calculations, p$K_a$ measurements, and X-ray crystal studies. However, addition of the second substituent does have a dramatic effect on the DNA binding of such compounds, as shown by binding assays and high-field NMR. The lowered binding energy and the loss of overlap geometry indicate intercalation to a very limited extent by each bound molecule. It is not possible to decide if this change occurs because of direct steric hindrance to intercalation by the second anilino substituent or whether its presence prevents the drug altering its minimum-energy conformation on binding.

Regardless of the reasons for the change in binding mode, it is accompanied by a dramatic loss of biological activity. This study provides further evidence that the nature and affinity of binding of drugs to DNA have a critical influence on their antitumor activity.

## Experimental Section

High-field NMR measurements were carried out in the Chemistry Department of the University of California at San Diego with a Varian HR300 spectrometer operating in the correlation mode; details are as in ref 4.

Rigid-geometry minimum-energy calculations were carried out on a VAX 11/780 computer at the Research Resource Computer Facility, Chemistry Department, University of California at San Diego, using programs provided by Dr A. T. Hagler.

Measurement of unwinding angles followed the method of Revet and employed covalently closed-circular DNA from PML21; details are given in ref 24.

For chemical analyses indicated by symbols of the elements, analytical results obtained for these compounds were within ±0.4% of the theoretical values. Analyses were performed by Professor A. D. Campbell, Microchemical Laboratory, University

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of Otago, Dunedin, New Zealand. Melting points were determined on an Electrothermal melting point apparatus with the manu- facturer's stem-corrected thermometer and are as read. NMR spectra were measured on a Varian EM-360A spectrometer.

**Synthesis of 4-Amino-3,5-dimethoxymethanesulfonanilide (12a) and 4-Amino-3,5-dimethylmethanesulfonanilide (12b) by the Method of Scheme I.** Experimental details are given for the dimethoxy isomer. Preparation of the dimethyl compound was essentially similar.

**N-Tosyl-2,6-dimethoxyaniline (6a).** Reaction of 2,6-di- methoxyaniline (40 mmol) and p-toluenesulfonyl chloride (42 mmol) in pyridine at 100 °C for 1 h gave the crude product, which was isolated by dilution with water, and crystallized from aqueous EtOH as needles, mp 165.5–166.5 °C (86%). Anal. $(C_{15}H_{17}NO_4S)$ C, H, N, S. The dimethyl isomer (6b) was similarly prepared in 88% yield: mp 136–138 °C (lit.[10] mp 136.5–137.5 °C).

**N-Tosyl-2,6-dimethoxy-4-nitroaniline (7a).** The above compound (40 mmol) was nitrated essentially by the method of Wepster.[10] Two crystallizations from EtOH gave pure product as yellow needles: mp 188.5–189.5 °C (71% yield); NMR [(C- D₃)₂CO] δ 2.47 (s, 3 H, CH₃), 2.78 (m, 1 H, NH), 3.78 (s, 6 H, OCH₃), 7.46 (s, 2 H, aromatic H), 7.56 (AB quartet, 4 H, $J = 8$ and 24 Hz, aromatic H). Anal. $(C_{15}H_{16}N_2O_6S)$ C, H, N, S.

The dimethyl isomer (7b) was similarly prepared in 73% yield: mp 167–168 °C (EtOH).

**2,6-Dimethoxy-4-nitroaniline (8a).** A suspension of the above tosyl compound (7.05 g, 20 mmol) in concentrated $H_2SO_4$ (d 1.84, 13.4 mL) and water (0.4 mL) was stirred at room temperature until homogeneous (3 h) and for a further 12 h. The mixture was poured into ice–NaOH to provide the crude amine, which was dried and recrystallized from benzene–ligroin and then aqueous EtOH to give orange prisms: mp 169–170 °C (77% yield); NMR [(CD₃)₂CO] δ 3.97 (s, 6 H, OCH₃), 5.23 (m, 2 H, NH₂), 7.50 (s, 2 H, aromatic H). Anal. $(C_8H_{10}N_2O_4)$ C, H, N. The dimethyl isomer (8b) was similarly prepared in 82% yield, mp 164–165 °C (lit.[14] mp 163.5–164.5 °C).

**N-(Trifluoroacetyl)-2,6-dimethyl-4-nitroaniline.** 2,6-Di- methyl-4-nitroaniline (1.66 g, 8.4 mmol) in pyridine (10 mL) was cooled to 0 °C, and CF₃COOH (0.92 mL, 12.0 mmol) was added, followed by PCl₃ (0.52 mL, 6.0 mmol). After 2 h at below 0 °C, followed by 2 h at 20 °C, the solvents were evaporated at low pressure, and the residue was triturated with water to give the crude product. Crystallization from EtOH gave needles, mp 194–195 °C (65% yield). Anal. $(C_{10}H_9F_3N_2O_3)$ C, H, N.

**N-(Trifluoroacetyl)-2,6-dimethyl-4-aminoaniline.** The above nitro compound (10 g, 38.2 mmol) was dissolved in boiling 65% aqueous EtOH and reduced by adding Fe powder (20 g), followed by FeCl₃ (0.5 mL of 15% solution). After 30-min reflux, CaCO₃ (1 g) was added, and the mixture was filtered. Evaporation of the filtrate and recrystallization of the residue from aqueous EtOH gave the amine, mp 172–173 °C (70% yield). Anal. $(C_{10}H_{11}F_3N_2O)$ C, H, N.

**4-[(Trifluoroacetyl)amino]-3,5-dimethylmethanesulfon- anilide.** Treatment of the above amine (4.0 g, 17.2 mmol) in pyridine (10 mL) at 0 °C with methanesulfonyl chloride (2.0 mL) for 2 h, followed by dilution with water, gave the crude product. Crystallization from boiling water gave plates, mp 134–135 °C (85% yield). Anal. $(C_{11}H_{13}F_3N_2O_3S)$ C, H, N.

**N-(Benzyloxycarbonyl)-2,6-dimethoxy-4-nitroaniline (9a).** 2,6-Dimethoxy-4-nitroaniline (8a: 20 mmol) was stirred in acetone (80 mL) with benzyl chloroformate (40 mmol) and MgO (50 mmol) for 12 h at room temperature. After this time, further quantities of benzyl chloroformate and MgO (10 mmol) were added, and the mixture stirred until TLC monitoring showed complete conversion (total time 18 h). The warmed mixture was filtered, and the solvents were removed to leave a product, which was crystallized twice from EtOH to give yellow needles, mp 194.5–195.5 °C (82% yield). Anal. $(C_{16}H_{16}N_2O_6)$ C, H, N.

Similar treatment of the dimethyl analogue (8b) with crys- tallization from aqueous EtOH gave N-(benzyloxycarbonyl)-2,6- dimethyl-4-nitroaniline (9b), mp 152–152.5 °C (64% yield). Anal. $(C_{16}H_{16}N_2O_4)$ C, H, N.

**N-(Benzyloxycarbonyl)-2,6-dimethoxy-4-aminoaniline (10a).** A solution of the above nitro compound (3.0 g, 10 mmol) in 65% aqueous EtOH (100 mL) and concentrated HCl (1 mL) was heated to reflux and treated portionwise with Fe powder (2.0 g). Iron salts were precipitated with ammonia, and the filtrate was evaporated to give crude product. Multiple crystallizations from aqueous EtOH gave pure product of mp 136–137 °C (73% yield). Anal. $(C_{16}H_{18}N_2O_4)$ C, H, N.

Similar treatment of the dimethyl compound (9b) gave the isomer (10b), which was crystallized from benzene–petroleum ether as colorless needles, mp 88–89 °C (86% yield). Anal. $(C_{16}H_{18}N_2O_2)$ C, H, N.

**4-[(Benzyloxycarbonyl)amino]-3,5-dimethoxymethane- sulfonanilide (11a).** A solution of the above amine (10a; 10 mmol) in pyridine (15 mL) was treated at 0 °C with methane- sulfonyl chloride (12 mmol). After 2 h at room temperature, the product was isolated by dilution with water and crystallized from aqueous EtOH as colorless needles, mp 185–186 °C (91% yield). Anal. $(C_{17}H_{20}N_2O_6S)$ C, H, N, S.

Similar treatment of the dimethyl analogue (10b) gave the product (11b) as needles from aqueous EtOH, mp 165.5–66.5 °C (84% yield). Anal. $(C_{17}H_{20}N_2O_4S)$ C, H, N, S.

**4-Amino-3,5-dimethoxymethanesulfonanilide (12a).** Hy- drogenolysis of the above carbobenzyloxy compound (11a) over Pd/C in MeOH, followed by crystallization of the product from benzene, gave colorless plates, mp 160–161 °C (87% yield). Anal. $(C_9H_{14}N_2O_4S)$ C, H, N, S.

Similar treatment of the dimethyl isomer gave 4-amino-3,5- dimethylmethanesulfonamide as needles from benzene, mp 148–148.5 °C (91% yield). Anal. $(C_9H_{14}N_2O_2S)$ C, H, N, S.

**Compounds 4 and 5 of Table I.** Coupling of the amines 12 with 9-chloroacridine to provide the amsacrine analogues were carried out in dry N-methylpyrrolidone, using a trace of dry HCl as catalyst, by warming the mixture until a red coloration indicated reaction.

The reaction with unhindered anilines usually proceeds at room temperature, but the dimethoxy derivative 12a required a tem- perature of 60 °C, while the even more hindered dimethyl de- rivative 12b required heating to 150 °C to initiate reaction.

After being held at the appropriate temperature for 15 min, the mixtures were cooled, and the product was precipitated by the addition of EtOAc. Recrystallization from MeOH–EtOAc gave the products 4 (yellow prisms, mp >360 °C) and 5 (orange needles, mp 261–263 °C) in good yield.

**Registry No.** 4, 86955-70-6; 5, 86955-71-7; 6a, 86955-72-8; 6b, 4703-15-5; 7a, 86955-73-9; 7b, 67083-22-1; 8a, 86955-74-0; 8b, 16947-63-0; 9a, 86955-75-1; 9b, 86955-76-2; 10a, 86955-77-3; 10b, 86955-78-4; 11a, 86955-79-5; 11b, 86955-80-8; 12a, 86955-81-9; 12b, 86955-82-0; CF₃CO₂H, 76-05-1; 2,6-dimethoxyaniline, 2734-70-5; p-toluenesulfonyl chloride, 98-59-9; N-(trifluoroacetyl)-2,6-di- methyl-4-nitroaniline, 86955-83-1; N-(trifluoroacetyl)-2,6-di- methyl-4-aminoaniline, 86955-84-2; 4-[N-(trifluoroacetyl)- amino]-3,5-dimethylmethanesulfonanilide, 86955-85-3; meth- anesulfonyl chloride, 124-63-0; benzyl chloroformate, 501-53-1; 9-chloroacridine, 1207-69-8.