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Dimeric and trimeric derivatives of the azinomycin B chromophore show enhanced DNA binding

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To explore the utility of the azinomycin B chromophore as a platform for the development of major-groove binding small molecules, we have prepared a series of 3-methoxy-5-methylnaphthalene derivatives containing diamine, triamine, and carbohydrate linker moieties. All bis- and tris-azinomycin derivatives are intercalators that display submicromolar binding affinities for calf-thymus DNA, as revealed by viscometry measurements and fluorescent intercalator displacement (FID) assays, respectively. Although the tightest binding ligand 1d (K_a=2.42 x 10^7 M^{-1}) has similar affinities for sequence diverse polynucleotides, competition binding studies with methylated phage DNA and known major and minor groove binding small molecules suggest that the tether moiety linking the naphthalene chromophores may occupy the major groove of DNA.

There are relatively few naturally occurring compounds that associate with the major groove of DNA. The vast majority of nucleic acid-binding natural products prefer to occupy the narrower minor groove, where hydrophobic and van der Waals interactions with the walls and floor of the groove are maximized.[1] The notable exceptions to this trend are the pluramycins, aflatoxins, azinomycin, leinamycin, and neocarzinostatin i-gb.[2] Intercalation of a planar delocalized-π system into the backbone of DNA is a consistent binding mode among all of these natural products, and with the exception of the aflatoxins, all of these substances also possess polar major-groove binding moieties. After a detailed study of the DNA binding interaction of azinomycin B, Gates suggested that the 3-methoxy-5-methyl naphthalene chromophore is a uniquely effective small, uncharged intercalator that accurately positions appended groups in the major groove of DNA.[3a] Coleman's subsequent studies on non-covalently binding Azinomycin derivatives indicated that intercalation did not occur for these compounds.[3b] However, Searcy's later investigation of similar naphthoate and naphthamide analogues of the azinomycin chromophore again provided evidence supporting an intercalative mode of binding.[3c] Given the synthetic accessibility of simple napthalene derivatives,[4] we envisioned that the azinomycin chromophore would be an ideal template for the development of sequence-specific major-groove binding small molecules. As a first step toward this goal, we have prepared a series of 3-methoxy-5-methyl naphthalene derivatives and evaluated their affinity for duplex DNA by ultraviolet and fluorescence spectroscopies.

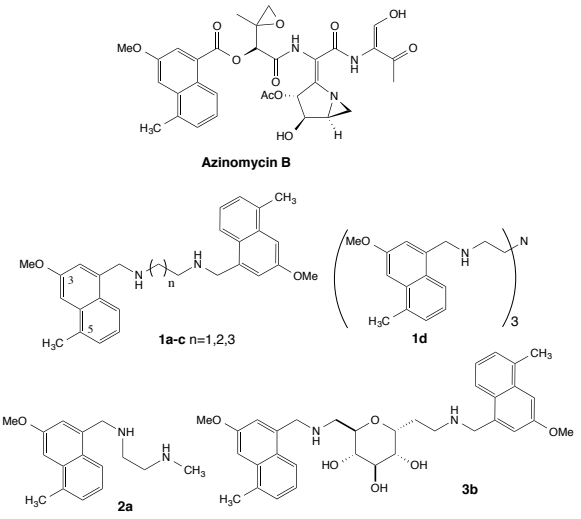


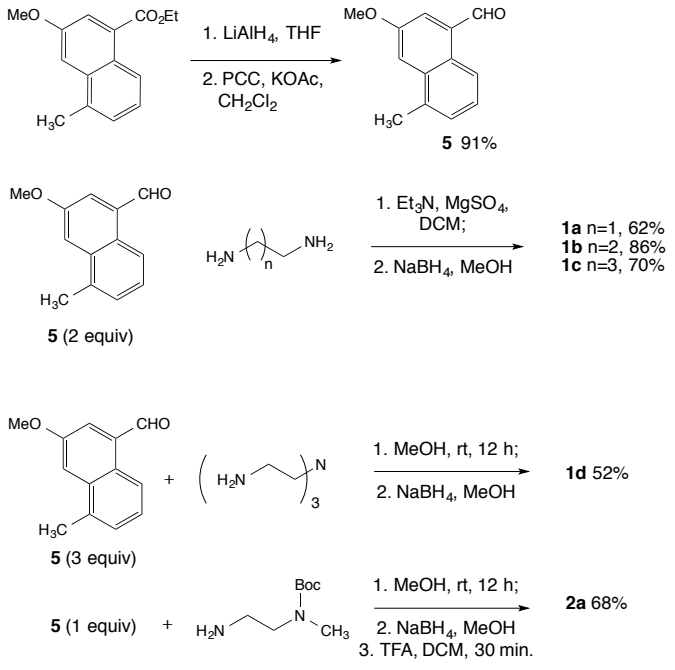
Figure 1. Designed ligands bearing the azinomycin chromophore.

DNA binding ligands possessing positively charged residues have a high affinity for duplex nucleic acids due to electrostatically favourable interactions with the negatively charged phosphate backbone and electronegative atoms in the major and minor grooves.[5] Thus the primary series of naphthalene derivatives (Figure 1, compounds **1** - **3**) targeted

for synthesis contain basic nitrogen atoms in the tethers linking the chromophores. Furthermore, dimerization of intercalating moeities has been shown to result in high-affinity DNA-binding ligands such as ditercalinium[6] and naphthalene diimides.[7] It was envisioned that ligands **1a-1d**, containing 2-, 3-, 4- and 5-atom tethers between the azinomycin chromophore units, would allow us to explore optimal distances between the aromatic moieties to achieve bis-intercalation.

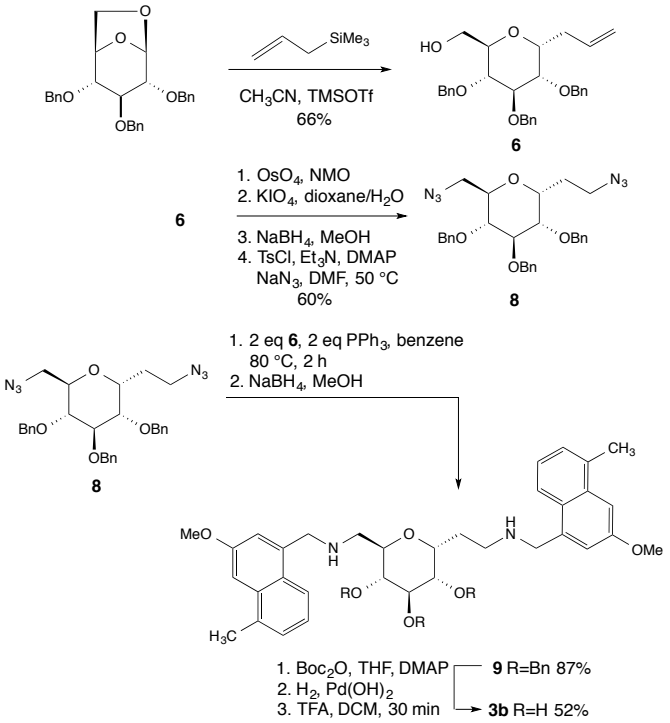
Waring[8] and McGee[9] have previously demonstrated that the presence of a carbohydrate moiety on an intercalating chromophore contributes positively and significantly to the binding association with DNA. It is also likely that carbohydrate moieties directed into the major groove would be able to participate in non-covalent interactions with functional groups on the edges of the AT and GC base pairs.[10] Ligand **3b** was therefore designed as an initial candidate for assessing high-affinity binding in the major groove of DNA.

Ethyl 3-methoxy-5-methylnaphthalene-1-carboxylate, readily available from 1-(2-methylphenyl)-2-propanone by Shibuya's protocol,[11] was our starting material for the synthesis of azinomycin chromophore derivatives. $LiAlH_4$ reduction of the ester gave the corresponding alcohol, which was oxidized with PCC to give the corresponding aldehyde **5** in 91% overall yield. Compounds **1a-1c** were prepared in 62-86% yields by combining 2 equivalents of naphthaldehye **5** with one equivalent of ethylene diamine, propylene diamine, or butylene diamine, respectively, in CH_2Cl_2 (Et_3N, $MgSO_4$, rt, 12h) followed by addition of CH_3OH and $NaBH_4$ (5 equiv) and stirring for 2 hours. Similarly, compound **1d** was prepared in 52% yield by combining 3 equivalents of naphthaldehyde **5** with one equivalent of tris(2-aminoethyl)amine in methanol (rt, 18 h), followed by addition of $NaBH_4$ (6 equiv) and stirring at rt for 2 hours. Combining instead *N*-Boc-*N*-methylenediamine with one equivalent of **5** in methanol (rt, 18 h), followed by addition of $NaBH_4$ (6 equiv, rt, 2 h) gave an intermediate carbamate, which was then treated with 1:1 TFA:DCM for 30 minutes and concentrated *in vacuo* to afford **2a** in 68% yield after purification.



Scheme 1. Synthesis of ligands **1a-d** and **2a**.

Compound **3b** was derived from *C*-allyl pyranoside **6**, which was prepared in turn according to the protocol of Trauner[12] by TMSOTf-promoted ring opening of 6-anhydro-2,3,4-tri-*O*-benzyl-β-D-glucose in the presence of allyl trimethylsilane. Oxidative cleavage (cat. OsO_4, NMO, acetone/H_2O; KIO_4, dioxane/H_2O) of the olefin and reduction ($NaBH_4$, MeOH, 2h, rt) provided an intermediate diol that was then treated with excess TsCl (Et_3N, DMAP, CH_2Cl_2, 2h, rt). The crude bis-tosylate was immediately combined with NaN_3 (6 equiv) in DMF (50 °C, 48 h) to provide bisazide **8** in 60% overall yield from **6**. Stirring **8**, PPh_3 (2 equiv) and napthaldehyde **5** (2 equiv) in benzene (1M, 80 °C, 2h), followed immediately by evaporation, dissolving in methanol, and treatment with $NaBH_4$ (4 equiv, 2h, rt) provided the intermediate bisamine **9** in 87% yield from **8**. Boc protection (3 equiv Boc_2O, Et_3N, DMAP, rt, 12 h) of the secondary amines, followed by catalytic hydrogenation (H_2, cat $Pd(OH)_2$, EtOH, rt, 12 h) and acidification (1:1 TFA/CH_2Cl_2, 30 min, rt) then gave rise to ligand **3b** in 52% yield from **9**.



Scheme 2. Synthesis of ligand **3a**.

Table 1. Estimation of CT-DNA association constants and stoichiometry for ligands **1a-d, 2a** and **3b** by ethidium displacement assays.

Ligand	K_{app} (x10^6 M^{-1})[a]	r_{bd}[b]
1a	7.19±0.92	4.2
1b	3.15±0.60	6.2
1c	5.00±0.46	7.0
1d	**24.2±1.5**	**9.1**
2a	0.47±0.03	4.0
3b	6.47±0.65	10.0

[a] K_{app} values obtained by the competitive ethidium displacement method (10 mM Tris-EDTA, pH=5.48), where $K_{app}=K_e$ x C_e/C_{50} and $K_e=2.1 \times 10^6 M^{-1}$.[13] [b] r_{bd} (ratio of CT DNA (bp):ligand) values were determined from the breakpoint of the curve in a plot of Δ fluorescence vs. CT DNA:ligand ratio, with the data obtained from the competitive ethidium displacement method.[14]

To compare the strengths of binding of compounds **1a-1d**, **2a**, and **3b** to calf thymus (CT) DNA, the competitive ethidium displacement technique was employed to obtain apparent association constants (K_{app})[13] and binding ratios (r_{bd}).[14] From this analysis (Table 1) the tightest binding synthetic ligand was the trisazinomycin derivative **1d** (K_{app}=2.42±1.5 x 10^7 M^{-1}), which has an apparent association constant more than 50-fold greater than that obtained for monoazinomycin derivative **2a** (K_{app}=4.7±0.3 x 10^5 M^{-1}). In addition, the bisazinomycin derivative **1a** (K_{app}=7.2±0.9 x 10^6 M^{-1}) has a 15-fold higher binding constant for CT DNA than **2a**, and since both ligands have two positively charged protonated nitrogen atoms at pH=5.48 and bind approximate the same number of base pairs (r_{bd}=~4.0), these results are suggestive of a bis-interacalative mechanism of association for the bis- and trisazino derivatives with DNA (*vide infra*). Interestingly, ligand **3b** (K_{app}=6.5±0.7 x 10^6 M^{-1}) showed no significantly enhanced binding relative to **1a-1c**, perhaps indicating that any stabilization due hydrogen-bond interactions between the glucosyl moiety linking the azinomycin chromophores and the DNA bases is offset either by steric crowding or entropic restriction of bond rotation in the intercalated complex.

To investigate the importance of electrostatic interactions[5] in the binding of the azinomycin chromophore derivatives to DNA, we evaluated the association of both **1a** and **1d** with CT DNA under high- and low-salt conditions using the ethidium displacement assay (See ESI). Increasing NaCl concentrations in the buffer (10 μM CT DNA in 10 mM Tris-EDTA, pH=5.48) from 0.01-1.0 M results in an approximately four-fold drop in **1d**'s DNA binding affinity; a similar lowering of DNA affinity was observed for **1a** between the concentrations of 0 mM and 100 mM NaCl. These data suggest that the positively charged protonated nitrogen atoms may be involved both in polar hydrogen bonding interactions in the grooves of DNA and in ionic interactions with the phosphate backbone.

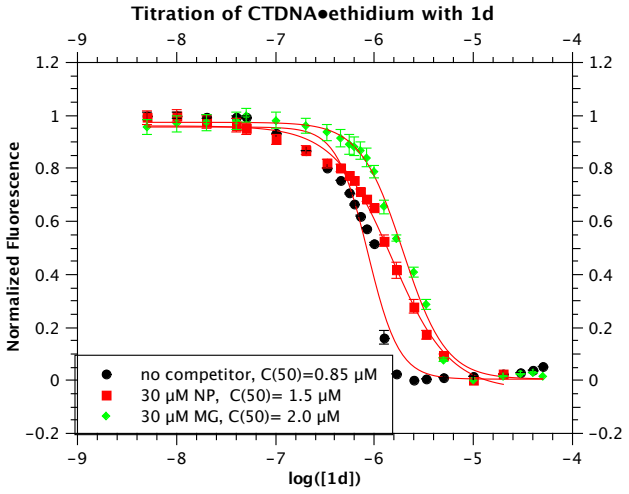


Figure 2. Binding isotherms for the titration of CT DNA (10 μM) and ethidium (10 μM) with **1d** (10 mM Tris-EDTA, pH=5.48) in the absence of competitor (black circles) or in the presence of netropsin (red squares) or methyl green (green diamonds). Black circles: no competitor, K_a = 2.47 ± 1.5 x 10^7 M^{-1}; red squares: 30 μM netropsin, K_a=1.40 ± 1.2 x 10^7 M^{-1}; green diamonds: 30 μM methyl green, K_a=1.05 ± 0.9 x 10^7 M^{-1}.

To evaluate the groove occupancy of the aliphatic tethering moiety connecting the intercalating naphthalene chromophores of our ligands, we investigated the displacement of ethidium bromide from CT DNA by **1d** in the presence of either the minor groove binding agent netropsin (NP, 30 μM) or the major groove binding agent methyl green (MG, 30 μM). The addition of netropsin resulted in an increase in the C50 value (concentration of **1d** required to achieve a 50% decrease in the fluorescence of ethidium) from 0.85 μM to 1.5 μM, corresponding to a 1.7-fold drop in the binding affinity of **1d** for CT DNA (Figure 2). The addition of methyl green instead resulted in a more pronounced increase in the C50 value from 0.85 μM to 2.0 μM, corresponding to an approximately 2.4-fold drop in the binding affinity of **1d** for CT DNA. These data suggest that the tethering moiety of **1d** may occupy either the major or minor grooves of DNA. Subsequent experiments (see below) provided additional evidence supporting major groove occupancy by **1d**.

The binding of **1d** to different DNA polynucleotides was also explored using the ethidium displacement assay (Table 2). The K_{app} values for the association of **1d** with polydG•polydC, polydA•polydT, and poly (dA•dT)$_2$ were in the range of 18 – 20 x 10^6 M^{-1}, indicating similar affinities for these binding sites. The higher value obtained for calf thymus DNA, however, likely indicates that **1d** prefers to bind heterogeneous sequences of natural B-form DNA. Since lambda phage DNA[20] is methylated in the major groove (*N6*-methyl adenine, *C5*-methyl cytosine), the three-fold lower binding constant observed for the association of **1d** with phage DNA vs. calf thymus DNA suggests that the aliphatic tethering moiety may occupy the major groove of DNA. In contrast, it was found that the minor groove binder netropsin binds to lambda phage DNA with a slightly *higher* apparent affinity than to calf thymus DNA (3.79±0.26 x 10^5 M^{-1} and 2.11±0.79 x 10^5 M^{-1}, respectively; see ESI).

Table 2. Evaluation of the binding of **1d** to different DNA polynucleotides by ethidium displacement assay.

DNA	K_{app} (x10^6 M^{-1})
polydG•polydC	18.4±0.6
Calf thymus	24.2±1.5
poly(dA•dT)$_2$	17.7±1.5
polydA•polydT	20.0±2.5
Lambda phage	8.02±0.7

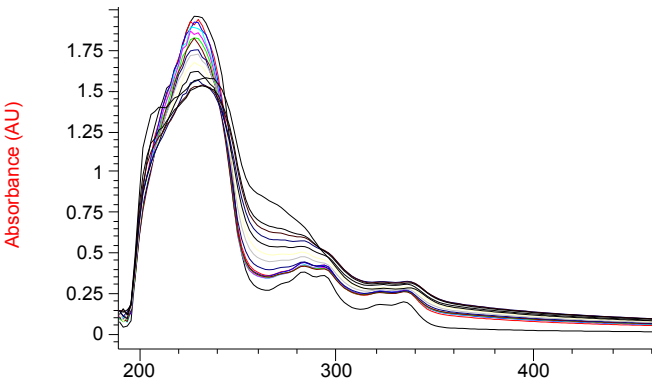


Figure 3. UV spectra of **1d** (25.0 μM in 10 mM Tris-EDTA, pH=5.48) in the presence of varying concentrations of CT-DNA: 0, 0.25, 0.5, 1.0, 2.0, 4.0, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 40.0, 50.0, 100 μM.

Monitoring the UV absorbance of **1d** at 228 nm upon titration with CT-DNA revealed a distinct hypochromic shift along with a slight redshift to 236 nm, observations again suggestive of an intercalative mode of binding (Figure 3).[15, 16] To confirm this, we performed viscometry studies for all synthetic ligands.[17] Increasing concentrations of **1d** (Figure 4) in the presence of CT-DNA resulted in an increase in solution viscosity comparable to that obtained with reference compound ethidium bromide, indicative of the helix lengthening and rigidification that occurs upon ligand intercalation. Similar results were obtained for ligands **1a**-**1c**, **2a**, and **3b** (see ESI). By comparison, the non-intercalative minor groove binder netropsin showed a relatively minimal increase in solution viscosity under the same conditions.

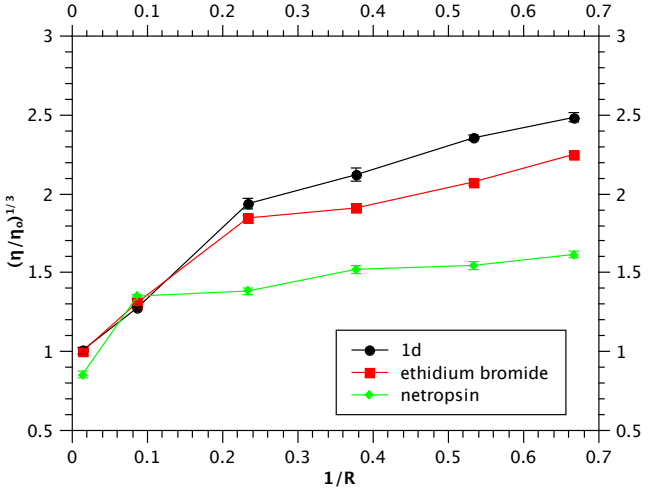


Figure 4. Effect of increasing amounts of **1d** (●), ethidium bromide (■) and netropsin (♦) on the relative viscosity of CT-DNA. *R*= [DNA(bp)]/[ligand]; **1d** (●): [CT-DNA] = 300 μM, [**1d**]: 1, 2, 4, 10, 18, and 26 μM; ethidium bromide (■): [CT-DNA] = 300 μM, [ethidium bromide]= 4, 26, 70, 113, 160, and 200 μM; netropsin (♦): [CT-DNA] = 300 μM, [netropsin]= 4, 26, 70, 113, 160, and 200 μM.

CD spectra of CT DNA recorded in the 220-310 nm region in the presence of increasing concentrations of **1d** revealed an initial increase in the intensities of both the helicity band at 240 nm and base-stacking band at 280 nm at low **1d**:DNA ratios, followed by a decrease in both bands at higher ligand concentrations (Figure 5). These observations, which mirror data previously recorded for the titration of CT DNA with ethidium bromide,[21] are indicative of an intercalative mode of DNA binding.

Molecular docking studies of ligand **1d**[23] with 5'-ATGCAT-3' (PDB 1x95) were performed using Autodock vina.[24] The energy-minimized bis-intercalation complex shows the chromophore tethering moiety occupying the major groove of DNA, with the ligand amino groups engaging in hydrogen bonding contacts with the edges of the sandwiched GC base pairs (Figure 6).

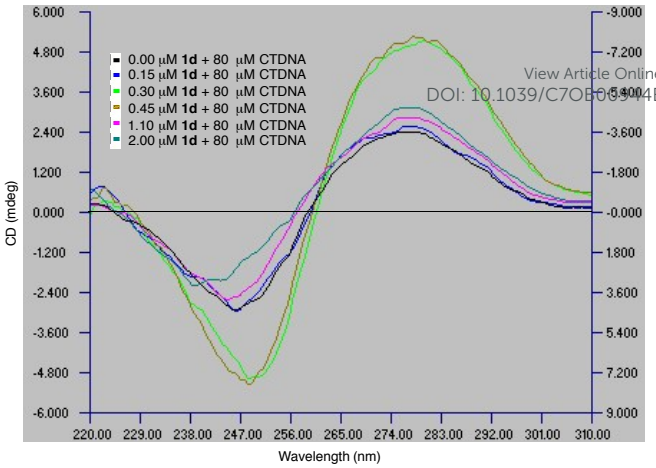


Figure 5. The 220-310 nm region of the CD spectrum of solutions of CT DNA (80 μM) in the absence (black line) and presence of various concentrations of **1d**: blue line, 0.15 μM **1d**; light green line, 0.30 μM **1d**; orange line, 0.45 μM **1d**; red line, 1.1 μM **1d**; dark green line 2.00 μM **1d**.

Conclusions

We have prepared a series of dimeric (**1a-1c**, **3b**) and trimeric (**1d**) derivatives of the azinomycin chomophore that show sub-micromolar binding affinities for duplex DNA. Viscometry data indicate that each of these ligands intercalate the backbone of DNA. Although the tightest binding derivative, **1d**, was shown to have low sequence selectivity, competitive binding studies with methyl green and lambda phage DNA indicate that association of this ligand with DNA may take place via the major groove. Since the CD and viscometry data for the interaction of **1d** with CT-DNA are very similar to that for ethidium bromide,[22] a bis-intercalation model for the association of **1d** with DNA is proposed, with the tethering moiety occupying the major groove.

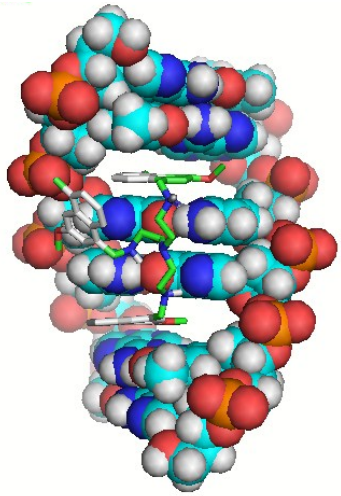


Figure 6. Model for bis-intercalation of ligand **1d** in the major groove of DNA sequence 5'-ATGCAT-3', generated by Autodock Vina using DNA (PDB 1x95) and **1d** minimized by Spartan 14 for Macintosh.[26]

We are currently exploring the cell permeability and cytotoxicity of these compounds (via MTT/MTS assays)[25] and the results of these studies will be reported in due course.

Acknowledgements

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