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**POTENTIAL DNA *BIS*-INTERCALATING AGENTS. SYNTHESIS AND ANTITUMOR ACTIVITY OF *N,N*-METHYLENEDI-4,1-CYCLOHEXANEDIYL- *BIS*(9-ACRIDINAMINE) ISOMERS<sup>‡</sup>**

**Gordon W. Gribble,<sup>a\*</sup> Michael D. Mosher,<sup>a</sup> Gary D. Jaycox,<sup>a</sup> Michael Cory,<sup>b</sup> and Terri A. Fairley<sup>b</sup>**

<sup>a</sup>Department of Chemistry, Dartmouth College, Hanover, NH 03755, USA  
ggribble@dartmouth.edu

<sup>b</sup>Division of Organic Chemistry, Burroughs Wellcome Company, Research Triangle Park, NC 27709, USA

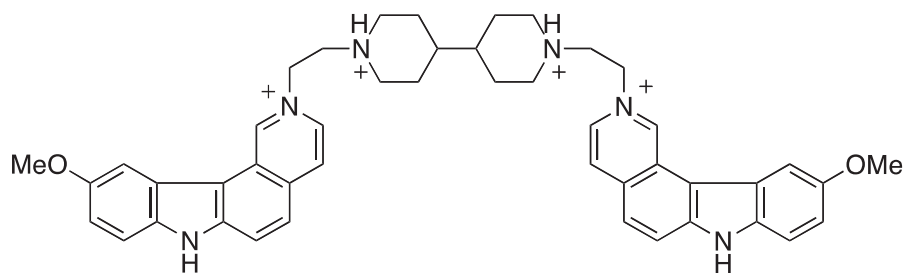
**Abstract** – We describe the synthesis and antitumor activity of three novel isomeric *bis*-acridines **14-16** possessing a non-aromatic, semi-rigid dicyclohexylmethylene tether.

## INTRODUCTION

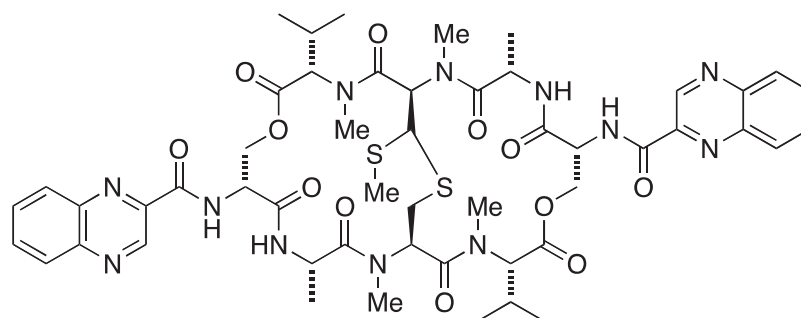
For several decades there has been considerable interest in the design, synthesis, and biological evaluation of potential DNA polyintercalators for the treatment of cancer and other diseases.<sup>1,2</sup> A prototypical compound is ditercalinium (**1**), which has entered clinical trials as an anticancer agent.<sup>3</sup> Moreover, nature has contributed a set of naturally occurring DNA *bis*-intercalators, the quinoxaline antitumor antibiotics, typified by echinomycin (**2**).<sup>4</sup> The planar acridine ring system is an excellent DNA intercalator and many investigations feature acridines and *bis*-acridines as potential anticancer and other agents.<sup>5,6</sup> At least one acridine drug, amsacrine (**3**), is used to treat human cancer.<sup>7</sup>

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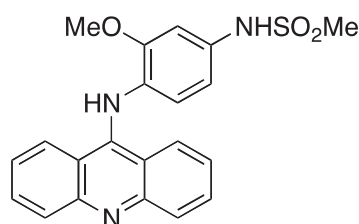
<sup>‡</sup> Dedicated to Professor Victor Snieckus in celebration of his 77th Birthday, for his countless contributions to organic synthesis, and for 50 years of friendship.



ditercalinium (1)

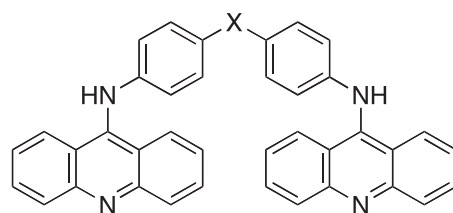
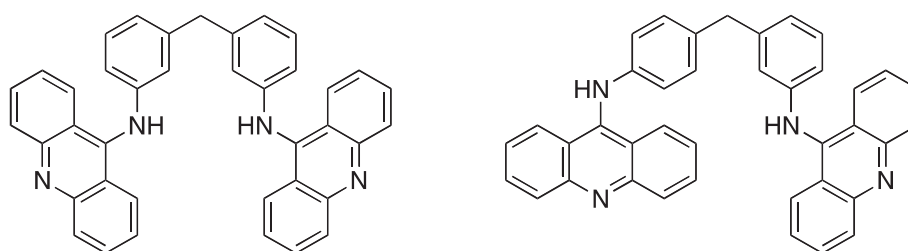


echinomycin (2)



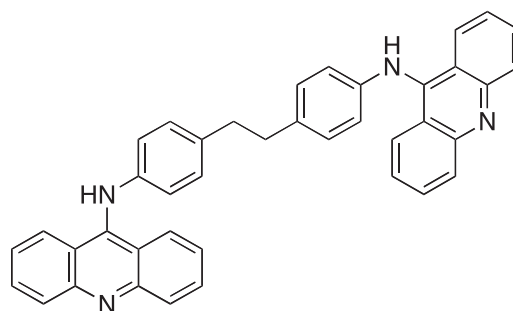
amsacrine (3)

In our previous work we described the synthesis and preliminary antitumor activity of a series of novel, conformationally restricted *bis*(9-aminoacridines) **4**, geometrically designed so as to encompass a two base-pair DNA sequence (four base-pair "sandwich").<sup>8</sup> Unlike conventional flexible tethers, our diphenylmethane tether was intended to minimize unfavorable entropic consequences and self-stacking interactions,<sup>9</sup> and also to deter "creeping" along the DNA, which drastically lowers ligand residence lifetimes at any one site.<sup>10</sup> DNA-ligand residence lifetimes are correlated with *in vivo* antitumor activity for many DNA intercalators.<sup>11</sup> In our screening of *bis*-acridines **4** *in vitro* in the L-1210 murine leukemia cell line, we found growth inhibition of  $ID_{50} = 0.081\text{--}0.090\ \mu\text{M}$ .<sup>8</sup> Consistent with manual docking of these and other *bis*-acridines with CPK models of DNA is that **5–8** are less active in the L-1210 screen ( $0.27\text{--}0.60\ \mu\text{M}$ ), as is 9-aminoacridine (**9**). Only **4**, with a single pivot atom and a *para*, *para*-linkage is able to achieve a *bis*-intercalative binding with the B form of DNA.

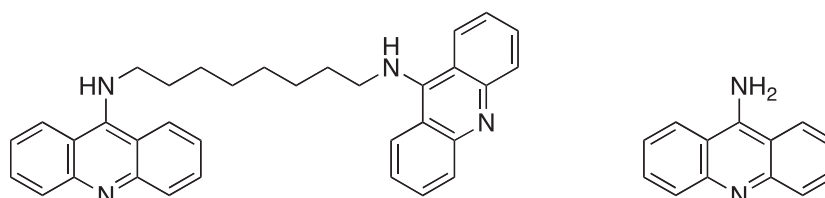
4 (X = CH<sub>2</sub>, O)

5 (0.27 μM)

6 (0.60 μM)



7 (0.42 μM)



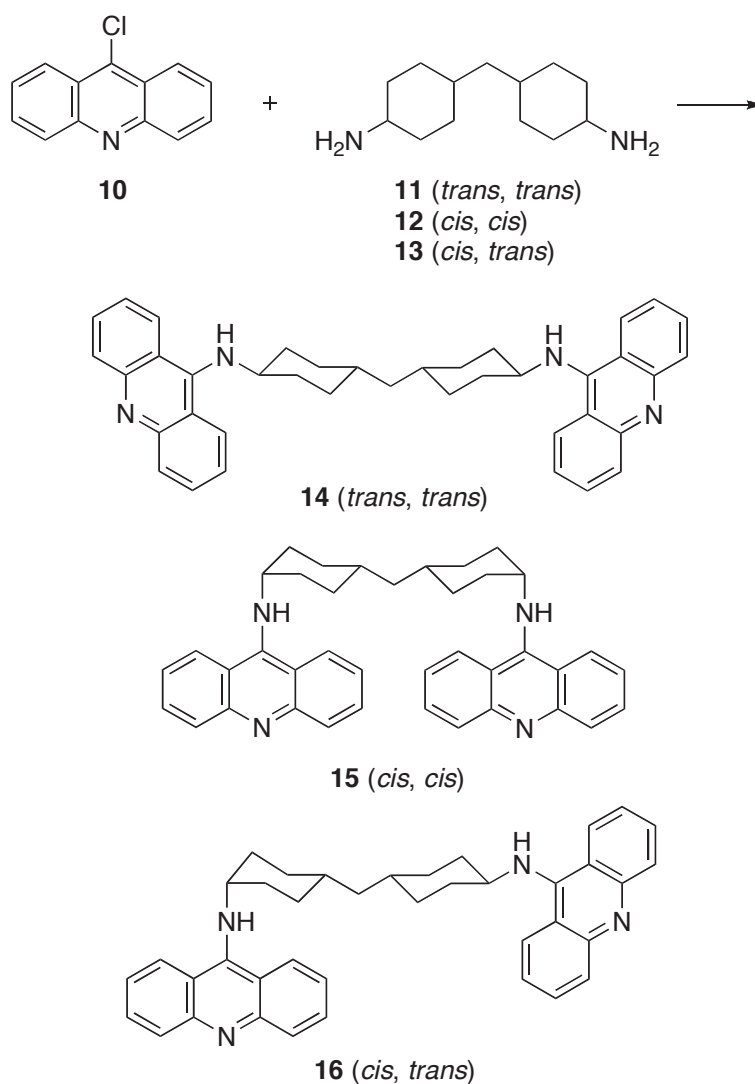
8 (0.40 μM)

9 (2.3 μM)

## RESULTS AND DISCUSSION

In an attempt to improve both the antitumor activity and solubility of this novel class of *bis*-acridines, we now describe the synthesis, purification, and preliminary antitumor activity and DNA binding of the isomeric *bis*(aminocyclohexyl)methane acridines **14–16** (Scheme 1). We anticipated that the more basic 9-alkylaminoacridines<sup>12</sup> would have improved water solubility over the less basic 9-arylaminoacridines, although we did not explicitly measure their water solubilities. The reaction of 9-chloroacridine (**10**) with the commercial mixture of 4,4'-dicyclohexyldiaminomethane isomers **11–13** (75% *trans, trans*; 5% *cis, cis*; and 20% *cis, trans*) gave the expected mixture of isomers **14–16**. However, since these could not be readily separated by silica chromatography, we separated and purified the individual diamines

**11–13.**<sup>13,14</sup> Thus, the commercial diamine mixture was distilled and fractionally crystallized to separate most of the dominant *trans, trans* **11**, which was acetylated to form the *bis*-acetamide. The remaining mixture of mainly **12** and **13** was acetylated to give the corresponding *bis*-acetamides, which were separated by fractional crystallization. All three air-stable *bis*-acetamides were recrystallized at least four times (to constant melting point), hydrolyzed with concentrated hydrochloric acid, and finally purified by distillation to give separate diamines **11–13**. These were distinguished by proton NMR according to the literature chemical shifts of the amino methine protons.<sup>14</sup> The diamines were allowed to react with 9-chloroacridine (**10**) in warm phenol to afford the individual *bis*-acridines **14–16**, in 70%, 28%, and 79% yields, respectively, which were characterized by elemental analyses and spectral data. Notably, each isomer could be distinguished by the chemical shift of the corresponding amino methine proton. Thus, *trans, trans* **14** shows a multiplet at 4.5 ppm (axial protons), *cis, cis* **15** at 4.75 ppm (equatorial protons), and *cis, trans* **16** shows multiplets at both positions. The clarity of this region in these NMR spectra confirms the purity of **14–16**.



**Scheme 1**

We were somewhat surprised to find that the *in vitro* antitumor activity of **14–16** against L-1210 murine leukemia is essentially identical (Table 1), and the weighted average ( $0.51 \mu\text{M}$ ) is close to that determined for the mixture of **14–16** ( $0.60 \mu\text{M}$ ). Also, the DNA melting temperature ( $\Delta T_m$ ) data reveal that all three isomers are equipotent in their binding to DNA. The magnitude of the  $\Delta T_m$  values suggests that the mode of binding is *bis*-intercalative. We were initially inclined to believe, from an examination of CPK space filling models of DNA and **14–16**, that the *cis, cis* isomer **15** would be the most effective *bis*-intercalator, but it seems clear from the  $\Delta T_m$  data that all three *bis*-acridines **14–16** are sufficiently flexible so as to *bis*-intercalate DNA, which can also adjust to the requirements of intercalation. The energy gained in DNA complexation must override the energy costs in forcing unfavorable boat and/or twist-boat cyclohexyl conformations for *bis*-intercalation to occur. 9-Aminoacridine (**9**) is included as a control compound. Analysis of the thermal denaturation studies in poly AT and poly GC DNA indicates that the *bis*-acridines have a greater impact on denaturation temperature, and potentially a larger binding constant, with the AT rich regions of genomic DNA. This impact is much greater for the purported *bis*-intercalators than for the mono-intercalating ethidium bromide as evidenced by the ratio of poly AT/poly GC  $\Delta T_m$  values (average 3.34 ratio for the *bis*-acridines vs. 1.87 ratio for ethidium bromide). This result lends further support that two intercalation events occur in the interaction of our *bis*-acridines with duplex DNA.

**Table 1.** Antitumor activity and DNA binding of *bis*-acridines **4–9**, **14–16**

<i>Bis</i> -Acridine	L-1210 Activity		$\Delta T_m, ^\circ\text{C}$	
	<i>In vitro</i> $\text{IC}_{50} \mu\text{M}^a$	Calf Thymus DNA	poly AT	poly GC
<b>4</b> (X = CH <sub>2</sub> )	0.081	16.7 ± 0.5	23.3 ± 0.52	7.9 ± 1.6
<b>5</b>	0.27	17.3 ± 1.8	21.5 ± 0.20	8.4 ± 0.9
<b>6</b>	0.60	14.5 ± 4.8	22.9 ± 5.5	5.5 ± 1.1
<b>7</b>	0.42	–	–	–
<b>8</b>	0.40	–	–	–
<b>9</b>	2.3	–	–	–
<b>14–16</b> (mix)	0.60	25.9 ± 4.1	19.7 ± 1.2	5.3 ± 1.1
<b>14</b>	0.50	–	–	–
<b>15</b>	0.44	–	–	–
<b>16</b>	0.56	–	–	–
Ethidium bromide	–	11.9 ± 1.1	9.9 ± 0.4	5.3 ± 0.7

<sup>a</sup>Dose that reduces by 50% the cell growth relative to controls, 72 h after drug exposure at 37 °C.

In conclusion, we have described the first examples of non-aromatic, semi-rigid tethered *bis*-acridines that display antitumor activity in the *in vitro* L-1210 murine leukemic screen. Given the expected increased basicity of the acridine ring nitrogen, it should be possible to prepare water-soluble salts and alkylated derivatives of **14–16** for *in vivo* screening. Such salts should be significantly more soluble than the free-bases.

## EXPERIMENTAL

Melting points were determined in open capillary tubes with a Büchi 510 melting point apparatus and are uncorrected. Nuclear magnetic resonance spectra were recorded on a Varian XL-300 multinuclear Fourier transform instrument and are reported in parts per million from tetramethylsilane on the  $\delta$  scale. The internal standard was trifluoroacetic acid, unless otherwise noted, which shows a resonance at 11.50  $\delta$ . Data are reported as follows: chemical shift [multiplicity (s = singlet, m = multiplet), integration]. Mass spectra were recorded either using fast atom bombardment (FAB) provided by the NIH Regional Facility located at the Massachusetts Institute of Technology (Cambridge, MA), or electron impact (20 eV) courtesy of Dr. M. Cory at Burroughs-Wellcome. Samples on which exact masses were measured exhibited no significant peaks at *m/e* greater than that of the parent. Combustion analyses were performed by Atlantic Microlabs (Norcross, AL). Attempts to remove THF solvent from **14–16** were unsuccessful. UV-visible spectra were recorded on a Hewlett-Packard 8451A Diode-Array Spectrophotometer from 300 nm to 820 nm. Extinction coefficients ( $\epsilon$ ) were not determined. The  $\lambda_{\max}$  values reported indicate the largest absorption in the range studied. Absorptions between 300 nm and 190 nm are not reported. Flash chromatography was performed on silica gel (Kieselgel 60, 230–400 mesh ASTM) from EM Science. Analytical thin layer chromatography (TLC) was carried out on 200  $\mu\text{M}$  Kieselgel 60 F<sub>254</sub> coated plastic sheets (Merck). The spots were visualized, after use of a mobile phase (ethyl ether, unless otherwise noted), with 254 nm UV light or by spraying with concentrated H<sub>2</sub>SO<sub>4</sub> and heating. All reactions were carried out under a nitrogen or argon atmosphere in oven-dried flasks unless otherwise stated. The calf thymus DNA and polynucleotides were obtained from commercial sources and quantified using spectroscopy. The highest grade commercial quality was used.

**9-Chloroacridine (10) (method A):** A magnetically stirred solution of *N*-phenylanthranilic acid (5.45 g, 26 mmol) in phosphorus oxychloride (100 mL, 1.07 mol) was treated with concentrated sulfuric acid (0.5 mL), then brought to reflux for 12 h. The solution was cooled to rt, added slowly to cracked ice (650 g), and made slightly alkaline with concentrated ammonium hydroxide. A light tan solid was collected by filtration, washed with water, and air dried for 24 h. Flash chromatography (silica gel/75% hexane, 20% THF, 5% triethylamine) afforded 4.67 g (84% yield) of **10** as fine yellow needles having one medium R<sub>f</sub>

spot by TLC; mp 117–119 °C (lit.<sup>15</sup> mp 119–120 °C); <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 8.50–8.10 (m, 4H), 7.95–7.50 (m, 4H); IR (THF): 3075, 1622, 1562, 1438, 1404, 1324, 1287, 1014, 831, 767, 647, 608 cm<sup>-1</sup>; Vis (CHCl<sub>3</sub>): λ<sub>max</sub> (ε) 360.5 nm (10,000); MS EI: *m/e* (relative intensity) 215 (33, M<sup>+</sup>+2), 213 (100, M<sup>+</sup>), 178 (21), 177 (16). Anal. Calcd for C<sub>13</sub>H<sub>8</sub>ClN: C, 73.07; H, 3.77; N, 6.55; Cl, 16.59. Found: C, 72.99; H, 3.80; N, 6.55; Cl, 16.58.

**9-Chloroacridine (10) (method B):** A magnetically stirred solution of 9-acridanone (4.01 g, 21 mmol) in phosphorus oxychloride (105 mL, 1.13 mol) was brought to reflux for 12 h. The solution was cooled to rt, added slowly to cracked ice (650 g), and made slightly alkaline with concentrated ammonium hydroxide. A light tan solid was collected by filtration, washed with water, and air dried for 24 h. Flash chromatography (silica gel/75% hexane, 20% THF, 5% triethylamine) afforded 3.97 g (89% yield) of **10** as fine yellow needles which was identical to material prepared by method A as judged by TLC, mp, and spectral data.

***N,N'*-(Methylenedi-4,1-cyclohexanediyl)bis[9-acridinamine] (mix of 14–16):** A magnetically stirred solution of 9-chloroacridine (**10**) (0.51 g, 2.39 mol) and 4,4'-diaminodicyclohexylmethane (0.13 g, 0.62 mmol) was warmed at 80 °C in phenol (20 g, 0.21 mol) for 4 h. After being cooled to rt, the solidified yellow-orange reaction mixture was dissolved in chloroform (100 mL) and extracted with 0.1 N NaOH (2 x 100 mL), water (3 x 100 mL), and brine (1 x 100 mL). The organic phase was separated, dried over anhydrous sodium sulfate, and then concentrated *in vacuo* to afford, after flash chromatography (silica gel/95% ethyl ether, 5% triethylamine), a bright yellow, hygroscopic solid. The solid was recrystallized from THF/hexane and dried *in vacuo* at 40 °C for 2 d to give 0.25 g (71%) of **14–16** as a yellow powder showing a single, very low R<sub>f</sub> spot by TLC; <sup>1</sup>H NMR (60 MHz, CF<sub>3</sub>COOD): δ 8.45–7.20 (m, 16H), 2.70–1.25 (m, 22H); IR (THF): 3578, 3508, 3352, 1631, 1565, 1526, 1428, 1356, 1077, 766 cm<sup>-1</sup>; Vis (CHCl<sub>3</sub>): λ<sub>max</sub> (ε) 398.5 nm (15,400); MS FAB: *m/e* 565 (MH<sup>+</sup>). Anal. Calcd for C<sub>39</sub>H<sub>40</sub>N<sub>4</sub>•1/2 H<sub>2</sub>O: C, 81.64; H, 7.20; N, 9.76. Found: C, 81.53; H, 7.39; N, 9.36. *In vitro* L1210: ID<sub>50</sub> = 0.09 μM (Vermont).

**Separation of *trans*, *trans*-(11), *cis*, *cis*-(12), and *cis*, *trans*-(13) isomers of 4,4'-diaminodicyclohexylmethane:** The commercial waxy paste was vacuum distilled (170–172 °C, 2–3 Torr) to give approximately 45 g of a colorless oil. The oil was dissolved in an equal volume of petroleum ether and allowed to crystallize. In this manner, colorless rods of **11** (10.9 g) were obtained. They were recrystallized from hexane twice more to give 6.0 g of **11** as rods and needles: mp 59–62 °C (lit.<sup>12</sup> mp 64–65.4 °C). The remaining petroleum ether contained the other two isomers as well as a

small amount of **11**. The solvent was removed *in vacuo* and the oil was acetylated: To a flask containing the liquid isomer (33.9 g) was slowly added, with stirring, water (40 mL) and glacial acetic acid (40 mL). The temperature was kept between 50–60 °C. Then after all of the isomer had dissolved, acetic anhydride (70 mL) was added and the solution stirred at 50–60 °C for 1 h. Water (80 mL) was added to the solution and after 15 min the resultant sandy ppt was filtered, washed with water, and labeled fraction A. The mother liquor was cooled to rt and after 2 h filtered, the sandy ppt washed with water, and labeled fraction B. The mother liquor was diluted with water (200 mL) and refrigerated overnight. The sandy crystals that formed were filtered, washed with water, and labeled fraction C. The mother liquor was further diluted to 1 L and after refrigeration overnight gave a small amount of sandy crystals, which were filtered, washed with water, and labeled fraction D. The mother liquor was concentrated to 100 mL on a steam bath, and the ppt that formed after cooling was filtered, washed with water, and labeled fraction E. Each fraction was recrystallized from ethanol-water four times (each time the mother liquor was concentrated and another fraction isolated and labeled), to give feathery white needles. The fractions (8.5 g) melting at 272–278 °C were determined to be, by comparison to the literature values, the *trans, trans*-diacetyl derivative. The fractions (13.0 g) melting at 225–228 °C were determined to be the *cis, trans*- derivative, and those (0.86 g) at 240–243 °C were determined to be the *cis, cis* derivative, by comparison to the literature<sup>13</sup> melting points. The free diamines of the *cis, trans* and *cis, cis* derivatives were then prepared. The *trans, trans* derivative was not transformed to the free diamine because it had been previously isolated from the crude mixture by fractional crystallization.

**4,4'-*cis, trans*-Diaminodicyclohexylmethane (13):** To the *cis, trans*-diacetyl derivative (4.42 g, 15.4 mmol) was added concentrated hydrochloric acid (50 mL) and the solution was refluxed for 24 h with stirring. Then, more hydrochloric acid (25 mL) was added and the solution was refluxed for 9 h. This was repeated once more, and finally the solution was allowed to stand at rt for 2 d. Neutralization of the clear solution with 50% aqueous sodium hydroxide gave a fluffy hydrate which was extracted with *n*-butanol (5 x 50 mL). The *n*-butanol extract was evaporated to a small volume and then washed with water to remove the salts. The remaining *n*-butanol was removed by distillation *in vacuo* to give a brown oil (3.0 mL). The oil was purified by distillation *in vacuo* (200 °C, 4 Torr) to give a colorless oil that when refrigerated overnight crystallized to give **13** as a waxy white solid (2.52 g, 79%): mp 26–28 °C (lit.<sup>13</sup> mp 35.7–36.9 °C). This was used without further purification.

***cis, cis*-4,4'-Diaminodicyclohexylmethane (12):** The *cis, cis*-diamine was prepared exactly as described above, to give after distillation *in vacuo* (200 °C, 4 Torr) an oil that crystallized on standing to give **12** as a waxy white solid (0.21 g, 79%): mp 57–59 °C (lit.<sup>13</sup> mp 60.5–61.9 °C). This was used

without further purification.

***N,N'*-trans, trans-Methylenedi-4,1-cyclohexanediyl-bis(9-acridinamine) (14):** To a mixture of warm (55 °C) phenol (10.5 g) and 9-chloroacridine (1.21 g, 5.66 mmol) was added *trans, trans*-4,4'-dicyclohexyldiaminomethane (**11**) (0.36 g, 1.74 mmol). The orange solution was stirred at 55–65 °C for 20 h. The reaction mixture was cooled to rt, taken up in CHCl<sub>3</sub> (100 mL), washed with 0.1 M NaOH (5 x 200 mL), water (2 x 100 mL), and brine (100 mL), and then dried (MgSO<sub>4</sub>). The orange organic phase was then filtered of drying agent and adsorbed onto silica gel. Short path flash chromatography (silica gel/hexane, Et<sub>2</sub>O, triethylamine, 5:20:1) gave **14** as orange-yellow powder. Recrystallization followed by drying *in vacuo* gave 0.68 g (70%) of **14** as a bright yellow powder with a single very low R<sub>f</sub> spot by TLC. Mp 240–242 °C; UV-vis (CHCl<sub>3</sub>) λ = 400 nm, 418 nm, 440 nm; <sup>1</sup>H-NMR: (TFA-d<sub>1</sub>) δ 8.3–7.5 (M, 16H); 4.5 (S, 2H); 2.6–1.2 (M, 20H); Anal. Calcd for C<sub>39</sub>H<sub>40</sub>N<sub>4</sub> + THF (C<sub>4</sub>H<sub>8</sub>O): C, 81.09%; H, 7.59%; N, 8.80%. Found: C, 80.57%; H, 7.62%; N, 8.75%; HRMS Calcd for (M<sup>+</sup>+1) C<sub>39</sub>H<sub>41</sub>N<sub>4</sub>: 565.3331; found, 565.3340.

***N,N'*-cis, cis-Methylenedi-4,1-cyclohexanediyl-bis(9-acridinamine) (15):** To a yellow solution of phenol (10 g) and 9-chloroacridine at 65 °C was added *cis, cis*-4,4'-dicyclohexyldiaminomethane (**12**) (0.15 g, 0.727 mmol). The mixture was stirred at 65–75 °C for 24 h. Then after cooling the orange-yellow mixture to rt, it was taken up in CHCl<sub>3</sub> (100 mL). The orange solution was washed with 0.1 M NaOH (5 x 100 mL), water (2 x 100 mL), and brine (100 mL), and dried (MgSO<sub>4</sub>). After adsorption onto silica gel, the mixture was subjected to short path flash chromatography (silica gel/Et<sub>2</sub>O, triethylamine (5%)) which gave a greenish yellow powder. Recrystallization (THF, hexane) and drying *in vacuo* afforded 0.115 g (28%) **15** as a bright yellow powder with a single, very low R<sub>f</sub> spot by TLC. Mp 232–234 °C; UV-vis (CHCl<sub>3</sub>): λ = 400, 418, 440 nm; <sup>1</sup>H-NMR (TFA-d<sub>1</sub>): δ 8.3–7.4 (M, 16H); 4.75 (S, 2H); 2.3–1.3 (M, 20H); Anal. Calcd for C<sub>39</sub>H<sub>40</sub>N<sub>4</sub> + 1 THF (C<sub>4</sub>H<sub>8</sub>O): C, 81.09%; H, 7.59%; N, 8.80%. Found: C, 80.75%; H, 7.65%; N, 8.63%; HRMS Calcd for (M<sup>+</sup>+1) C<sub>39</sub>H<sub>41</sub>N<sub>4</sub>: 565.3331; found, 565.3354.

***N,N'*-cis, trans-Methylenedi-(4,1-cyclohexanediyl)-bis(9-acridin-amine) (16):** To a warm (65 °C) solution of phenol (10 g) and 9-chloroacridine (1.08 g, 5.05 mmol) was added *cis, trans*-4,4'-dicyclohexyldiaminomethane (**13**) (0.26 g, 1.26 mmol). The orange-yellow solution was stirred at 65 °C for 20.5 h before cooling to rt. The solidified mixture was then taken up in CHCl<sub>3</sub> (100 mL), washed with 0.1 M NaOH (5 x 100 mL), water (2 x 100 mL), and brine (100 mL), and then dried (MgSO<sub>4</sub>). After adsorption onto silica gel, the mixture was subjected to short path flash chromatography

(silica gel/Et<sub>2</sub>O, triethylamine (5%)) which afforded a yellow powder. Recrystallization (THF/hexane) and drying *in vacuo* overnight gave 0.56 g (79%) of **16** as a bright yellow powder, showing a single very low R<sub>f</sub> spot by TLC. Mp 197–198 °C; UV-vis (CHCl<sub>3</sub>): λ = 400, 418, 440 nm; <sup>1</sup>H-NMR (TFA-d<sub>1</sub>): δ 8.4–7.4 (M, 16H); 4.8 (S, 1H); 4.45 (S, 1H); 2.4–1.2 (M, 20H); Anal. Calcd for C<sub>39</sub>H<sub>40</sub>N<sub>4</sub> + ½ THF (C<sub>4</sub>H<sub>8</sub>O): C, 82.15%; H, 7.33%; N, 9.44%. Found: C, 81.89%; H, 7.30%; N, 9.77%; HRMS Calcd for C<sub>39</sub>H<sub>41</sub>N<sub>4</sub> (M<sup>+</sup>+1): 565.3331; found, 565.3342.

**Procedure for determining L1210 activity:** The individual compounds were tested by Professor Miles P. Hacker, Department of Pharmacology, University of Vermont. L1210 cells (10<sup>5</sup>) maintained as suspension cultures in McCoy's 5A medium (containing 10% horse serum, glutamine, penicillin, and streptomycin) were exposed to known concentrations (0.01, 0.1, and 10 μg/mL in DMSO) of the compounds in an atmosphere of 10% carbon dioxide and 90% air at 37 °C. After 72 h of continuous exposure the population of each culture was determined by a Coulter Counter (Model ZBF, Hialeah, FL) and compared to the control. The ratio of exposed cell population to control cell population was used to calculate the percent cell growth relative to control.<sup>8</sup> This was used to calculate the IC<sub>50</sub> value (the drug concentration required to inhibit cell growth by 50% of control).

**Procedure for determination of ΔT<sub>m</sub> values:** The thermal denaturation studies were done by the method of Cory<sup>16</sup> on a Varian 2290 UV-visible spectrophotometer with a heating rate of 18 °C/h. The five cuvettes were on a 2 min cycle time with a 5 s dwell time. ΔT<sub>m</sub> were calculated from the printout using the difference between the compound T<sub>m</sub> and the DNA. The calf thymus DNA had a T<sub>m</sub> of 56.8 °C.

## ACKNOWLEDGEMENTS

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