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# Synthesis and DNA binding studies of bis-intercalators with a novel spiro-cyclic linker

Yongjun Chu, Vincent Lynch and Brent L. Iverson*

*Department of Chemistry and Biochemistry, The University of Texas at Austin, 1 University Station A5300, Austin, TX 78712, USA*

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**Abstract**—Threading polyintercalation has been demonstrated as a unique DNA binding mode in which a polyintercalating moiety threads back and forth through the DNA double helix. This binding topology necessitates linkers residing in both the minor and major grooves in an alternating fashion. In the present work, two novel, rigid, cis and trans oriented spiro-cyclic linkers were synthesized as potential groove binding elements in the context of threading bis-intercalation. Analysis of dissociation kinetics indicated that the cis oriented dimer has dramatically slower dissociation from poly(dGdC) and calf thymus (CT) DNA compared to the trans oriented dimer and a linear dimer control.


## 1. Introduction

Recognizing specific DNA sequences using synthetic molecules has been an exciting research area for over two decades due to the potential for controlling gene expression, creating new therapeutics, and exploration of molecular recognition in general. Several approaches to DNA recognition have been developed. A particularly successful strategy involves minor groove binding, exemplified by the naturally occurring netropsin, distamycin, beneril, and their analogues.[1] Based on these molecules, programmable sequence-specific binding has been achieved with the polyamides developed by Dervan and co-workers.[2] Importantly, molecules of this class have been shown to modulate gene function in vitro[3] and even in vivo,[4] although cellular uptake appears to be a considerable challenge. A second approach to programmable DNA recognition involves triplex formation in the major groove.[5]

Recently, our group has developed a novel threading polyintercalator approach based on the 1,4,5,8-naphthalenetetracarboxylic diimide (NDI) unit first identified as a threading intercalator by Wilson and co-workers.[6] Our multiple diimide polyintercalators are designed to bind specific DNA sequences, threading through both the major and minor grooves, an unprecedented feature for a DNA binding molecular scaffold.[7–9] It has been demonstrated that two different threading bis-intercalators can bind to specific DNA sequences with their linkers in the minor and major grooves, respectively.[7,8] Significantly, NMR structural analysis of a designed threading tetraintercalator was shown to have a predicted threading binding topology in which linkers alternated between the minor and major grooves, much as a snake might climb a ladder.[9] However, in this same analysis, the major groove binding linker exhibited significant disorder, leading to the conclusion that a properly designed, rigidified linker design could greatly improve binding specificity and affinity.

The present paper describes the design and synthesis of a rigidified linker intended to be a scaffold for future linker designs. Preliminary DNA binding studies are reported in the context of threading bis-intercalation.

## 2. Results and discussion

### 2.1. Linker design

The flexibility of our first generation linkers likely limits overall specificity as well as possible programmable recognition of different sequences of DNA. Specific recognition based on shape and/or hydrogen bonding complementarity may be compromised by the flexibility inherent in the unconstrained peptide chains. A conformationally restricted linker scaffold, of the proper size and geometry, should be able to improve not only specificity of DNA recognition, but overall binding affinity as well.[10] The important caveat here is that a rigidified scaffold should be readily synthesizeable and have the potential to be derivatized with various

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* Corresponding author. Tel.: +1 512 471 3949; fax: +1 512 232 2382; e-mail: biverson@mail.utexas.edu

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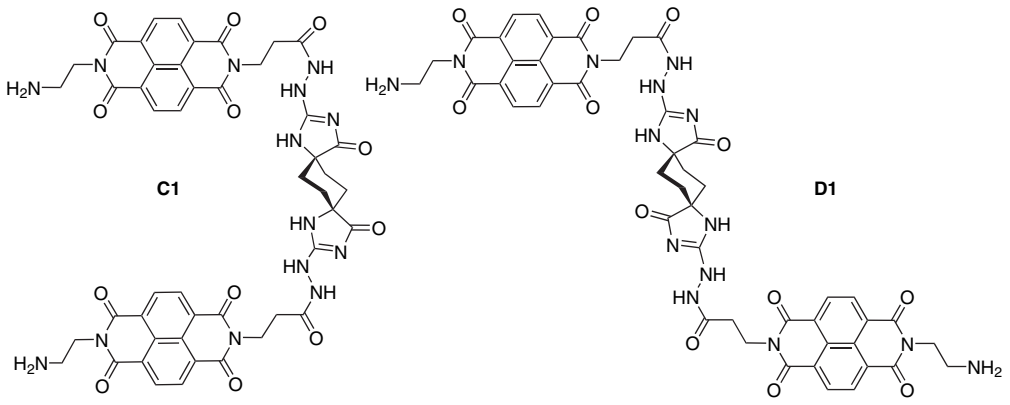



**Figure 1.** The structures of two dimers, **C1** and **D1**, containing cis and trans spiro-cyclic linkers, respectively.

functional groups capable of interacting with specific sites on the DNA bases.

Shown in Figure 1 are two novel rigid scaffolds.[11] The two heterocycles are intended to (1) lock the central cyclohexane ring into a chair conformation and (2) form hydrogen bonds with bases on the floor of a DNA groove. Next generation designs would involve derivatization of the central six-membered ring to achieve alternative specificities. Computer modeling indicates that these rigid scaffolds could be compatible with binding in either the minor or major grooves, increasing the potential applications of the design (Fig. 2). The same modeling indicated that the cis isomer should be more compatible with the curvature of DNA compared to the trans derivative.

## 2.2. Synthesis

**2.2.1. trans-Spiro linker.** A Bucherer–Bergs reaction gave the two bis-spirohydantoins shown (**1** and **2**) in excellent yields (Schemes 1 and 2).[12] Both structures were found to be in the trans geometry based on NOESY spectra (not shown). The kinetic and/or thermodynamic reasons for the exclusive formation of the trans geometry (i.e., no cis products were observed) are currently unknown. The *trans*-1, 4-diamino-1,4-dicarboxylic acid dimethyl ester (**3**) was synthesized in good yield. The structure of intermediate **5** was confirmed by X-ray crystallography (Fig. 3).

Scheme 3 describes the conversion of diamino dimethyl carboxylate **6** into the desired trans linker **10**. The easy removal of benzoyl group makes benzoyl isothiocyanate an ideal reagent to form a precursor to the *trans*,bis-spirothiohydantoin **8**. Using mild base, intermediate **8** was derived from cyclization in 75% yield. The structure of **8** was confirmed by X-ray crystallography (Fig. 4). Subsequent methylation and hydrazine substitution gave the desired trans linker **10** in good overall yield.

**2.2.2. cis-Spiro linker for C1.** Since the cis oriented bis-hydantoins could not be made via Bucherer–Bergs reaction when the two carbonyl groups of 1,4-cyclohexane were cyclized simultaneously, the two cyclization reactions were carried out separately. For this purpose, mono-protected 1,4-cyclohexanedione was used as shown in Scheme 4.

The Bucherer–Bergs reaction was used with the mono-protected 1,4-cyclohexanedione to give mono-spirohydantoin **11**. NMR analysis confirmed that **11** contained the NH group and CO group in axial and equatorial orientations, respectively. Alkylation gave compound **12**, whose geometry was confirmed by X-ray crystallography (Fig. 5). Acidic deprotection gave compound **13** in high yield. Based on the difference between *A*-values of $-NH_2$ and –CN, we anticipated that a Strecker reaction would give a cis and trans mixture of amino monohydantoins.[13] However, following a Strecker reaction on intermediate **13**, cyclization using chlorosulfonyl isocyanate and 1 M HCl gave us two bis-spirohydantoins **16** and **17**. Their NOESY spectra (not shown) suggested that both were of the trans geometry.

The fact that no cis isomer was found under the above reaction conditions might indicate that the reaction occurring on one carbonyl group directs the reaction pattern on the other. In addition, the unfavorable interaction between NH and $NH_2$ may contribute as well. Interestingly, in the case of an analogous five-member ring system, a cis and trans mixture was formed.[14]

A second attempt was made to obtain the cis structure based on a Diels–Alder strategy (Scheme 5) via key intermediate **24**. Starting from **18**, which can be made in a few steps,[15] DIBAl-H reduction at 0 °C and Diels–Alder reaction with DEAD gave the compound **20** in a modest yield due to competing side reactions such as aromatization of **19** and the ene reaction.[16] Enough material could be generated to continue the synthesis, but this step is nevertheless going to be optimized to hopefully improve the yield. Following protection of the hydroxyl groups, reductive cleavage using Na in ammonia gave compound **22** in high yield. The two hydroxyl groups were successfully oxidized using basic $KMnO_4$.

Shown in Scheme 6 are the final steps to make the cis linker **30** analogous to the procedures used in the trans linker synthesis. The cis geometry of bis-spirothiohydantoin intermediate **28** was confirmed by X-ray crystallography (Fig. 6).

The corresponding trans and cis NDI dimers **D1** and **C1** were synthesized in high yield using PyBop and HOBT as the coupling reagents (Scheme 7) to attach previously reported NDI derivatives to the trans and cis linkers.[17]

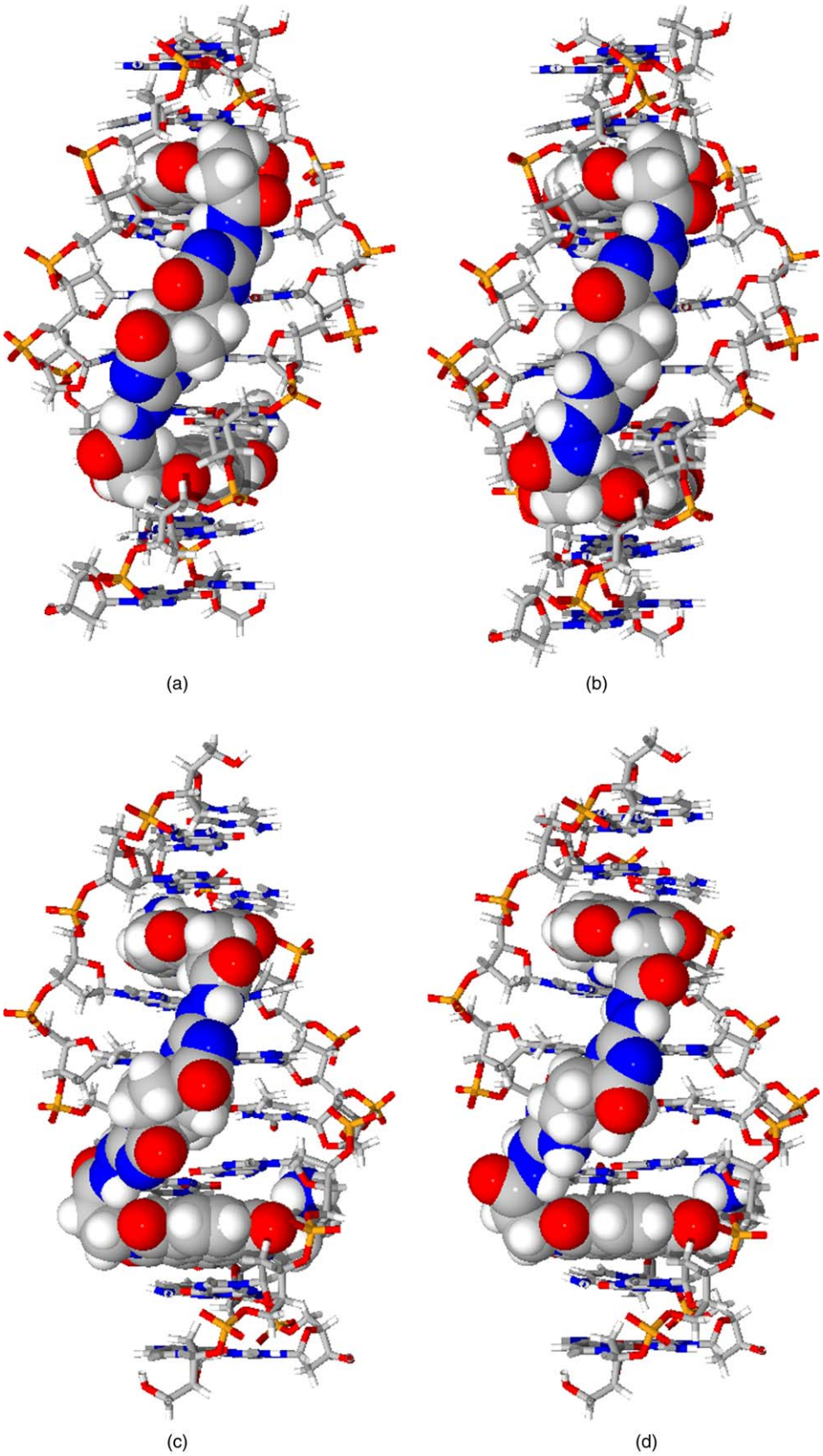
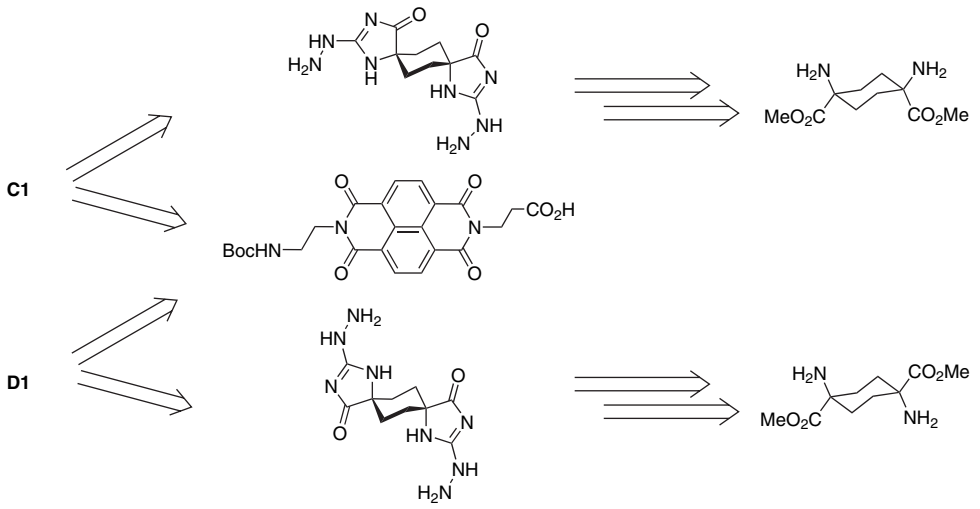
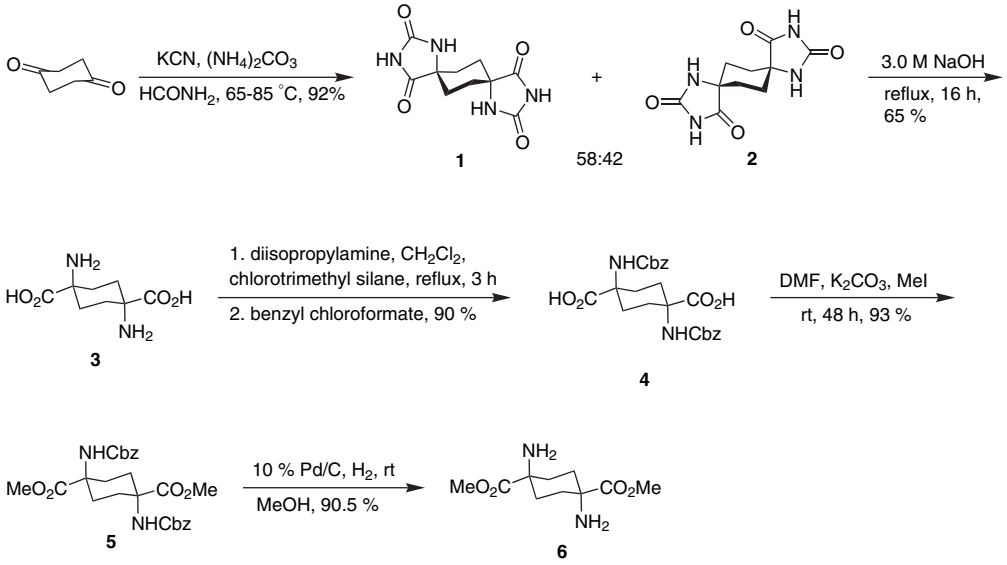



**Figure 2.** View from the minor grooves (a and b) and major grooves (c and d) of the **C1** (left) and **D1** (right) bis-intercalator/d(CGGTACCG)$_2$ complexes.




**Scheme 1.** Retrosynthetic analysis of bis-intercalators **C1** and **D1**.




**Scheme 2.**

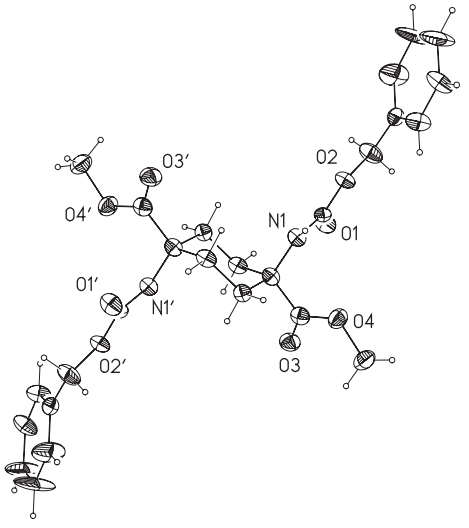
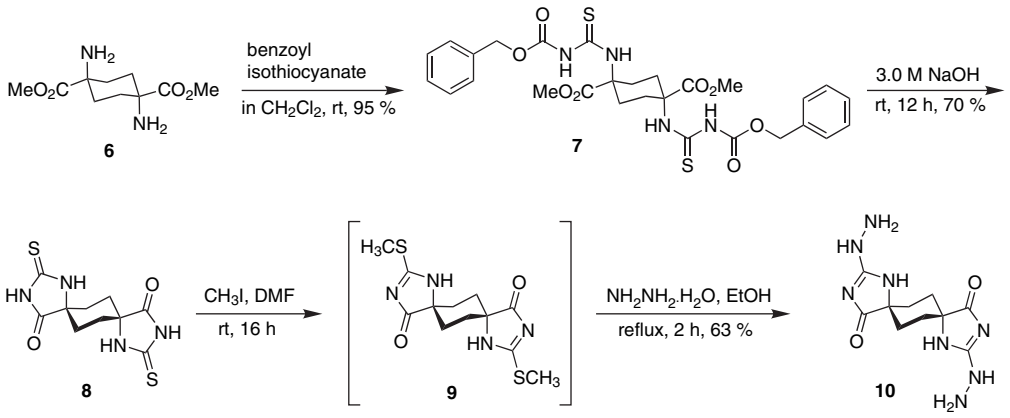



**Figure 3.** Displacement ellipsoid diagram for **5**.

## 2.3. Kinetics studies

As a preliminary investigation into the interaction of **D1** and **C1** with DNA, dissociation rates were determined for CT DNA, poly(dGdC) and poly(dAdT). These values were compared to similar data obtained with a previously reported dimer containing a flexible peptide linker (referred to a **$G_3K$**).[8] For NDI bis-intercalators, dissociation rates are generally sufficiently slow to allow monitoring using a spectrophotometer without the need for a stopped-flow apparatus. Dissociation rate measurements were carried out with 2% SDS in the buffer to sequester the dissociated intercalators following dissociation from the DNA.[17] Control studies indicated that the dimers did not reassociate with the DNA under these conditions. All dissociation profiles were adequately described by one phase exponential decay, $A=a_0 \exp(-kt)+b_0$.[18]




**Scheme 3.**

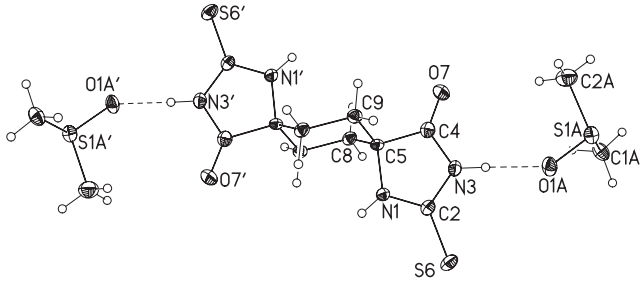



**Figure 4.** Displacement ellipsoid diagram for **8**·DMSO.

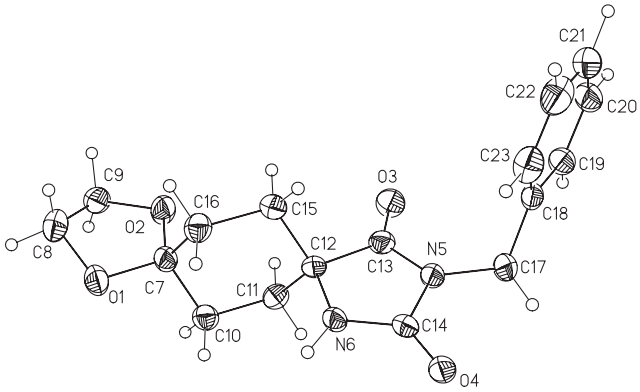
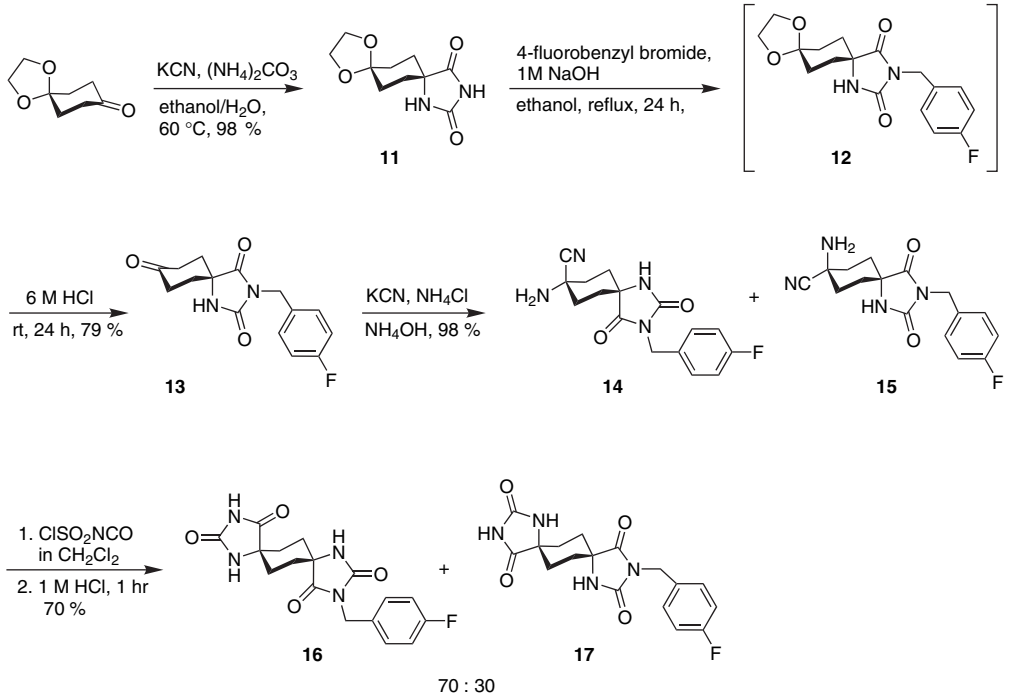
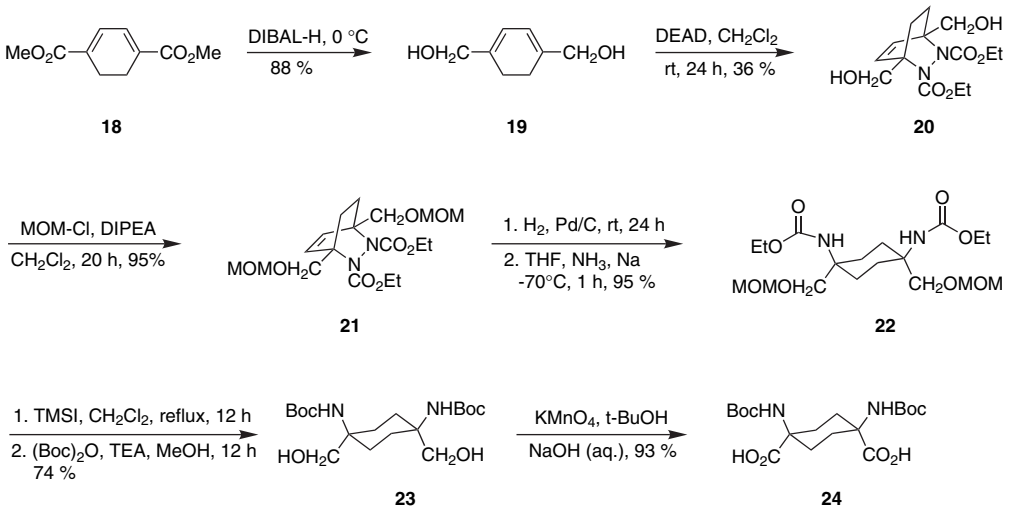



**Figure 5.** Displacement ellipsoid diagram for **12a** (an analogue of **12**).

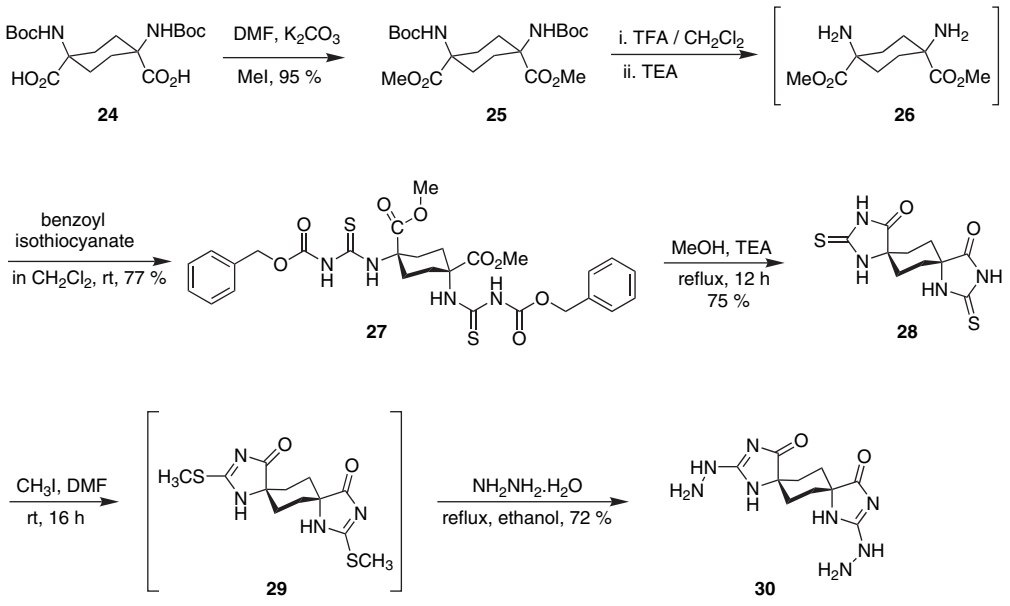
The dissociation rate data reveal that all dimers exhibit a dramatic preference for poly(dGdC) over poly(dAdT) (Table 1 and Supplementary data), a trend seen with previously reported NDI based intercalation.[17] The dissociation half-lives measured using poly(dGdC) for all of the dimers extend over 10 min, with **C1** exhibiting a 50 min half-life. In contrast, the dissociations of the three dimers from poly(dAdT) were almost complete within 1 min, with the cis dimer **C1**




**Scheme 4.**




**Scheme 5.**




**Scheme 6.**

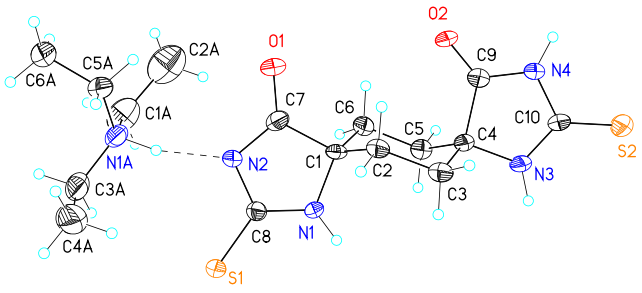
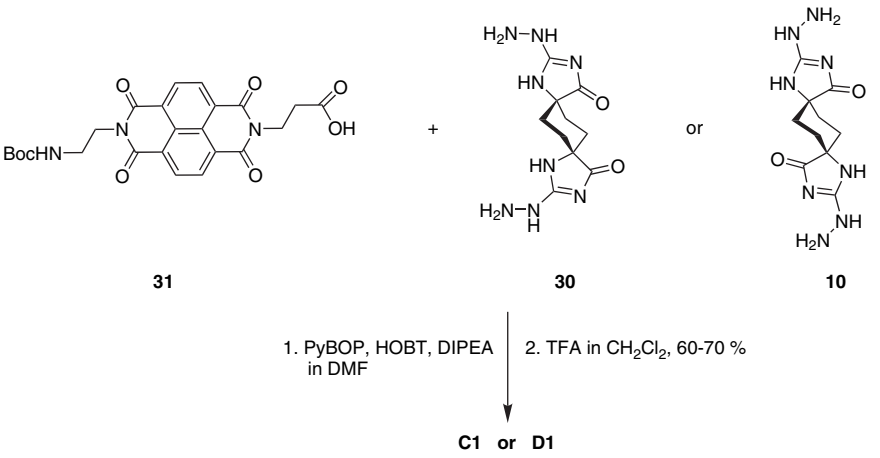



**Figure 6.** Displacement ellipsoid diagram for **28**·N($CH_2CH_3$)$_3$.

exhibiting the shortest half-life. Taken together, our data indicate that the cis dimer **C1** binds with greatest overall affinity to poly(dGdC), and with the greatest overall ability to discriminate between poly(dGdC) and poly(dAdT).

## 3. Conclusion

Two novel threading bis-intercalators containing cis and trans oriented spiro-cyclic linkers were successfully synthesized, although preparation of the cis derivative proved to be a considerable challenge. Data from dissociation kinetics suggested that the DNA binding behavior of the cis oriented dimer **C1** is different from that of the trans oriented dimer **D1** and linear dimer **$G_3$K** based on studies with poly(dGdC), poly(dAdT), and CT DNA. The cis dimer **C1** shows the slowest off-rate toward poly(dGdC) and the fastest off-rate with poly(dAdT). The most straightforward interpretation of these results is that by constraining the rigidified linker in the appropriate cis geometry, both binding affinity and specificity have been enhanced compared to our flexible linker designs. DNAse I footprinting experiments and an NMR structural analysis are currently being carried out to identify the binding modes and specificities of **C1**.




**Scheme 7.** Solution phase dimer synthesis.

**Table 1**. Dissociation rates and half-life times

| DNA type | D1 | | C1 | | $G_3K$ | |
|---|---|---|---|---|---|---|
| | $k$ ($s^{-1}$) | $t$ (min) | $k$ ($s^{-1}$) | $t$ (min) | $k$ ($s^{-1}$) | $t$ (min) |
| Poly(dAdT) | 1.02 | 0.682 | 2.86 | 0.243 | 1.10 | 0.629 |
| Poly(dGdC) | 0.0528 | 13.1 | 0.0138 | 49.9 | 0.0471 | 14.7 |
| CT DNA | 0.51 | 1.4 | 0.13 | 5.3 | 0.37 | 1.9 |

## 4. Experimental

### 4.1. General

All chemicals were purchased from Aldrich, Acros, or Novabiochem. Dry solvents were either purchased or dried using common laboratory techniques. All chemical reactions were carried out in oven-dried glassware under an argon atmosphere. Silica gel 60 $F_{254}$ glass-based plates (Merck) were used for TLC. Flash chromatography was carried out using ICN SiliTech 32-63D 60 Å silica gel. All NMR spectra were recorded on Varian 300 or 500 MHz instruments. $CDCl_3$ ($\delta_H$=7.24 and $\delta_C$=77.0 ppm) or DMSO-$d_6$ ($\delta_H$=2.49 and $\delta_C$=39.5 ppm) or $D_2O$ ($\delta_H$=4.67 ppm) were used as solvents. Assignment of $^{13}C$ signals is based on $^1H$ and $^{13}C$-correlated 2D NMR spectra.

**4.1.1. 1,3,9,11-Tetraaza-dispiro[4.2.4.2]tetradecane-2,4,10,12-tetraone (1,α) and (2,β).** To a 40 mL Ace pressure tube were added 1,4-cyclohexanedione (1.12 g, 0.01 mol), potassium cyanide (1.43 g, 0.022 mol), and ammonium carbonate (4.8 g, 0.05 mol). Formamide (35 mL) was then added and the pressure tube was tightly closed. The tube was heated at 60 °C for 24 h, then at 85 °C for another 24 h with vigorous stirring. Vacuum filtration at room temperature gave a white solid after rinsing with cold water and ethanol. The filtrate was treated with enough mixed ion exchange resin (AG® 501-X8 (D) Resin, 20–50 mesh, BIO-RAD) to extract remaining cyanide anions. The crude solid was washed with $CH_2Cl_2$ (3×20 mL), giving a pure solid product (2.32 g, 92%): $^1H$ NMR (500 MHz, DMSO-$d_6$): $\delta$ 10.57 (br s, 2H), 8.53 (s, 1H, H1′ of **1**), 8.18 (s, 1H, H1′ of **2**), 2.10–2.05 (m, 4H, equatorial of **2**), 1.99–1.91 (m, 4H, equatorial of **1**), 1.68–1.63 (m, 4H, axial of **2**), 1.62–1.57 (m, 4H, axial of **1**); $^{13}C$ NMR (125.7 MHz, DMSO-$d_6$): $\delta$ 177.4 (C4′ of **2**), 177.3 (C4′ of **1**), 155.8 (C2′ of **1**), 155.7 (C2′ of 2), 60.3 (C1 and C4 of **1**), 59.4 (C1 and C4 of **2**), 28.8 (C2, C3, C5, and C6 of **2**), 28.4 (C2, C3, C5, and C6 of **1**); HRMS-CI calcd for $C_{10}H_{13}N_4O_4$ $[M+H]^+$: 253.0937, found 253.0926.

**4.1.2. *trans*-1,4-Diamino-cyclohexane-1,4-dicarboxylic acid (3).** The dihydantoins **1** and **2** (1.16 g, 0.0046 mol) were suspended in 12 mL of 3 M NaOH aqueous solution and the mixture was heated at reflux (120 °C) while stirring for 16 h. The reaction mixture then was cooled to room temperature and filtered through a fritted glass filter. The filtrate was brought to pH 2 with 3 M HCl at 0 °C and a white precipitate formed. After 10 min at 0 °C, the mixture was filtered through a fine fritted glass filter. The solid was washed with copious cold deionized water and dried at 50 °C under vacuum oven over 12 h, yielding the desired product **3** (0.59 g, 65%): $^1H$ NMR (300 MHz, $D_2O$+NaOD): $\delta$ 1.75 (d, 4H, equatorial, $J$=9 Hz), 1.21 (d, 4H, axial, $J$=9 Hz); $^{13}C$ NMR (75.5 MHz, $D_2O$+NaOD, uncorrected): $\delta$ 184.8 (COOH), 57.5 (C1 and C4), 31.1 (C2, C3, C5, and C6). No useful mass data were available due to insolubility of this compound in common solvents.

**4.1.3. *trans*-1,4-Bis-(benzyloxycarbonylamino)-1,4-cyclohexanedicarboxylic acid (4).** To a 50 mL flask equipped with a magnetic stir bar and rubber septum was added diamino acid **3** (0.6 g, 0.003 mol). The solid was suspended in 20 mL of $CH_2Cl_2$. Diisopropylamine (1.72 mL, 0.0099 mol) was added with stirring. Chlorotrimethylsilane (1.71 mL, 0.0135 mol) was then added slowly, the rubber septum was replaced with a reflux condenser, and the solution was heated at reflux under nitrogen for 4 h. The condenser was removed and replaced with another rubber septum. The flask was cooled in an ice bath, and benzyl

chloroformate (Cbz-Cl) (0.81 mL, 0.006 mol) was added in one portion via syringe. The stirred solution was allowed to warm and stirred at room temperature overnight. The mixture was concentrated to a pink syrup, dissolved in 100 mL 1.0 M aqueous NaOH, and transferred to a separatory funnel. The aqueous solution was washed with ether (2×100 mL). The ether washes were combined and back extracted with water (2×200 mL). The aqueous layers were combined and acidified to pH 2 with 3 M HCl at 0 °C. White solid formed and was filtered through a fine fritted glass filter. The solid was washed with cold water then air-dried to give the desired product **4** (1.27 g, 90%): $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 12.40 (br s, 2H, COOH), 7.56 (s, 2H, 2NH), 7.36–7.31 (m, 10H, aromatic), 5.00 (s, 4H, 2C$H_2$Ph), 1.92–1.82 (m, 8H, 4$CH_2$ of central ring); $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta$ 176.0 (COOH), 155.5 (CONH), 137.0, 128.3, 127.8, and 127.7 (aromatic), 65.1 ($CH_2$Ph), 57.4 (C1 and C4), 26.4 (C2, C3, C5, and C6); HRMS-CI calcd for $C_{24}H_{27}N_2O_8$ [M+H]$^+$: 471.1767, found 471.1774.

**4.1.4. *trans*-1,4-Bis-(benzyloxycarbonylamino)-1,4-dimethoxycarbonyl-cyclohexane (5).** Dicarboxylic acid **4** (1.955 g, 4.1 mmol) was dissolved in dry DMF (33 mL) in a 100 mL round bottom flask, and then anhydrous $K_2CO_3$ (1.72 g, 12.4 mmol) was added with vigorous stirring. MeI (0.65 mL, 10 mmol) was added slowly by syringe and the reaction was stirred at room temperature for 48 h. $H_2O$ (120 mL) was added at the end of the reaction and the product precipitated. Filtration in a fine fritted glass filter followed by washing with cold water gave the desired product (1.9 g, 93%, $R_f$=0.25 in 1/49 $CH_3OH/CH_2Cl_2$): $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 7.76 (s, 2H, 2CONH), 7.37–7.30 (m, 10H, aromatic), 5.01 (s, 4H, 2C$H_2$Ph), 3.56 (s, 6H, 2$CH_3$), 1.92–1.82 (m, 8H, 4$CH_2$ of central ring); $^{13}$C NMR (75.5 MHz, DMSO-$d_6$): $\delta$ 174.7 ($CO_2CH_3$), 155.5 (CONH), 137.0, 128.4, 127.8, and 127.7 (aromatic), 65.2 ($CH_2$Ph), 57.6 (C1 and C4), 52.0 ($CH_3$), 26.4 (C2, C3, C5, and C6); HRMS-CI calcd for $C_{26}H_{31}N_2O_8$[M+H]$^+$: 499.2080, found 499.2075.

**4.1.5. *trans*-1,4-Diamino-1,4-dimethoxycarbonyl-cyclohexane (6).** Ester **5** (0.5 g, 1 mmol) was suspended in 20 mL methanol in a 50 mL three-neck round bottom flask, which was pre-saturated with dry Ar. Pd/C (10%, 0.15 g) was added slowly. The solution was degassed, placed under hydrogen atmosphere (10 psi), and stirred for 1.5 h. The reaction mixture was filtered through Celite on a fine fritted glass filter. The filtrate was evaporated at room temperature and then dried in vacuo. The dried solid was used directly for the next step without further purification (0.21 g, 90.5%, $R_f$=0.33 in 2/3 $CH_3OH/CH_2Cl_2$): $^1$H NMR (300 MHz, pyridine-$d_5$): $\delta$ 3.59 (s, 6H, 2$CH_3$), 2.51–2.45 (m, 4H, equatorial), 2.03 (br s, 4H, 2$NH_2$), 1.63–1.57 (m, 4H, axial); $^{13}$C NMR (75.5 MHz, pyridine-$d_5$): $\delta$ 178.6 ($CO_2CH_3$), 56.9 (C1 and C4), 51.8 ($CH_3$), 30.5 (C2, C3, C5, and C6); HRMS-CI calcd for $C_{10}H_{19}N_2O_4$ [M+H]$^+$: 231.1345, found 231.1347.

**4.1.6. *trans*-1,4-Bis-((*N*-benzoyl(thiocarbamoyl)amino)-1,4-dimethoxycarbonyl-cyclohexane (7).** Diamine **6** (0.115 g, 0.5 mmol) was dissolved in dry $CH_2Cl_2$ (10 mL) in a 50 mL round bottom flask. Benzoyl isothiocyanate (0.15 mL, 1.1 mmol) was added by syringe with stirring. The solution became cloudy after stirring for a few hours. The reaction was terminated after 24 h by adding another 10 mL of $CH_2Cl_2$ to dilute the mixture and the solid was isolated by filtration using a fine fritted glass filter (0.26 g, 95%, $R_f$=0.35 in 1/1 $CH_3OH/CH_2Cl_2$): $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 11.56 (s, 2H, 2(C═O)NH(C═S)), 11.43 (s, 2H, 2NH(S═C)), 7.99–7.93 (m, 4H, aromatic), 7.68–7.64 (m, 2H, aromatic), 7.58–7.54 (m, 4H, aromatic), 3.61 (s, 6H, 2$CH_3$), 2.57–2.52 (m, 4H, equatorial), 1.97–1.93 (m, 4H, axial); $^{13}$C NMR (75.5 MHz, DMSO-$d_6$): $\delta$ 179.8 (C═S), 172.0 ($CO_2$Me), 169.0 (CONH), 133.3, 131.9, 128.8, and 128.5 (aromatic), 70.1 ($CH_2$Ph), 60.0 (C1 and C4), 52.3 ($CH_3$), 26.4 (C2, C3, C5, and C6); HRMS-CI calcd for $C_{26}H_{29}N_4O_6S_2$ [M+H]$^+$: 557.1529, found 557.1525.

**4.1.7. *trans*-Cyclohexane-1, 4-bis-spiro-5′,5″-(2′,2″-thiohydantoin) (8).** Isothiocyanate **7** (0.51 g, 0.9 mmol) was dissolved in 10 mL of 3 M NaOH aqueous solution. After stirring for 5 min, white precipitate formed in the flask. Stirring was continued for 16 h. The reaction was terminated by adding 1.0 M HCl to bring the solution pH to neutral. After being stirred for additional 1 h the mixture was filtered and rinsed with cold water to give the crude product. The product was purified by crystallization in DMSO (0.18 g, 70%): $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 11.65 (br s, 2H, 2(C═O)NH(C═S)), 10.70 (br s, 2H, 2(C═S)NH), 2.02 (d, 4H, equatorial, $J$=9.3 Hz), 1.69 (d, 4H, axial, $J$=9.3 Hz); $^{13}$C NMR (75.5 MHz, DMSO-$d_6$): $\delta$ 181.4 (C═S), 178.0 (C═O), 63.8 (C1 and C4), 27.7 (C2, C3, C5, and C6); HRMS-CI calcd for $C_{10}H_{13}N_4O_2S_2$ [M+H]$^+$: 285.0480, found 285.0488.

**4.1.8. *trans*-2,10-Dihydrazino-1,3,9,11-tetraaza-dispiro[4.2.4.2]tetradeca-1,9-diene-4,12-dione (10).** Thiohydantoin **8** (1.0 g, 3.5 mmol) was dissolved in anhydrous DMF (10 mL) with gentle heating in 25 mL round bottom flask. Methyl iodide (0.88 mL, 14 mmol) was then added via syringe. The flask was clear and light yellowish for the first few hours, and then some pale yellowish precipitate formed inside. After stirring the mixture for 12 h at room temperature, the solvent was removed at room temperature in vacuo. The dry solid was used directly for the next step due to its presumed moisture sensitivity. Solid methylsulfanyl hydantoin **9** was dissolved in 30 mL of ethanol in a round bottom flask. Hydrazine monohydrate (0.85 mL, 17.6 mmol) was added dropwise to the mixture. The clear solution was heated at reflux for 2 h, forming a white precipitate. The flask was cooled down to room temperature and vacuum filtration gave a white powder that was further dried in vacuo (0.62 g, 63%): $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 8.90 (br s, 2H, 2NH), 8.28 (s, 2H, 2N$H$N$H_2$), 4.49 (s, 4H, 2$NH_2$), 1.99 (d, 4H, equatorial, $J$=8.6 Hz), 1.34 (d, 4H, axial, $J$=8.6 Hz); $^{13}$C NMR (75.5 MHz, DMSO-$d_6$+TFA): $\delta$ 178.0 (C═O), 158.9 (NH–C═N), 64.0 (C1 and C4), 29.1 (C2, C3, C5, and C6); HRMS-CI calcd for $C_{10}H_{17}N_8O_2$ [M+H]$^+$: 281.1474, found 281.1465.

**4.1.9. 9,12-Dioxa-1,3-diaza-dispiro[4.2.4.2]tetradecane-2,4-dione (11).** To a 40 mL pressure tube, 1,4-cyclohexanedione monoethylene acetal (1.56 g, 0.01 mol), potassium cyanide (0.65 g, 0.01 mol), and ammonium carbonate (2.4 g, 0.025 mol) were added followed by 35 mL ethanol/water

(v/v=1/1). The reaction tube was tightly capped and heated at 65 °C for 24 h with stirring. The reaction mixture was cooled down to 0 °C using an ice bath. Approximately half of the solvent was removed by vacuum evaporation and the solid was isolated by vacuum filtration. The filtrate was treated with mixed ion exchange resin (AG® 501-X8 (D) Resin, 20–50 mesh, BIO-RAD) to extract the remaining KCN. The solid product was rinsed with dichloromethane and dried in vacuo (2.21 g, 98%): $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 10.55 (br s, 1H, (C=O)NH(C=O)), 8.44 (s, 1H, C=ONH–C), 3.86 (s, 4H, O($CH_2$)$_2$O), 1.87–1.54 (m, 8H, central ring); $^{13}$C NMR (75.5 MHz, DMSO-$d_6$): $\delta$ 178.2 (C4′), 156.3 (C2′), 106.8 (C1), 63.7 and 63.6 ($OCH_2$-$CH_2O$), 61.0 (C4), 31.3 (C2 and C6), 29.8 (C3 and C5); HRMS-CI calcd for $C_{10}H_{15}N_2O_4$ $[M+H]^+$: 227.1032, found 227.1028.

**4.1.10. 3-(4-Fluoro-benzyl)-1,3-diaza-spiro[4.5]decane-2,4,8-trione (13).** Hydantoin **11** (1.13 g, 0.005 mol) was suspended in 1.0 M NaOH solution (5 mL) and ethanol (5 mL) and the mixture was heated at reflux for 15 min. To this solution was added 4-fluorobenzyl bromide (0.59 mL, 0.0048 mol) dropwise through the top of a reflux condenser. The mixture was heated at reflux for 24 h and allowed to cool in ice bath. The resulting precipitate was washed with water and recrystallized from ethanol to give pure product **12**. Compound **12** was then dissolved in 3 M HCl (10 mL) and ethanol (10 mL). The clear solution was stirred at room temperature for 16 h. The reaction was terminated by adding 3 M NaOH to adjust the pH of the solution to 7.5. $CH_2Cl_2$ (4×40 mL) was used to extract the product from the mixture. The $CH_2Cl_2$ layer was dried ($Na_2SO_4$), followed by vacuum evaporation to yield the crude product. Flash silica gel chromatography (1/19 $CH_3OH/CH_2Cl_2$) gave the product **13** (1.14 g, 79%, $R_f$=0.33 in 1/19 $CH_3OH/CH_2Cl_2$): $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 9.07 (s, 1H, NH), 7.31–7.26 (m, 2H, aromatic), 7.18–7.12 (m, 2H, aromatic), 4.54 (s, 2H, C$H_2$Ph), 2.55–2.48 (m, 2H, axial on C2 and C6), 2.38–2.32 (m, 2H, equatorial on C2 and C6), 2.17–2.08 (m, 2H, axial on C3 and C5), 1.95–1.89 (m, 2H, equatorial on C3 and C5); $^{13}$C NMR (75.5 MHz, DMSO-$d_6$): $\delta$ 208.0 (CO), 175.8 (C1′), 161.0 (d, aromatic C–F, $^1J_{CF}$=242 Hz), 155.5 (C3′), 132.9 (aromatic), 129.3 (d, aromatic *C*–(C–C–F), $^3J_{CF}$=8.8 Hz), 115.3 (d, aromatic *C*–(C–F), $^2J_{CF}$=21.5 Hz), 59.8 (C4), 40.4 (*C*$H_2$Ph), 36.3 (C2 and C6), 32.9 (C3 and C5); HRMS-CI calcd for $C_{15}H_{16}N_2O_3F$ $[M+H]^+$: 291.1145, found 291.1141.

**4.1.11. 8-Amino-3-(4-fluoro-benzyl)-2,4-dioxo-1,3-diaza-spiro[4.5]decane-8-carbonitriles (14 and 15).** To a 100 mL round bottom flask were added 4-fluorobenzyl hydantoin cyclohexanone **13** (0.6 g, 2.06 mmol), potassium cyanide (0.28 g, 4.3 mmol), ammonium chloride (0.24 g, 4.4 mmol), ammonium hydroxide (15 mL), and ethanol (30 mL). The flask was stirred at room temperature for 16 h. The clear reaction solution was then transferred to a separatory funnel and extracted with $CH_2Cl_2$ (4× 50 mL). The aqueous layer was treated with mixed ion exchange resin (AG® 501-X8 (D) Resin, 20–50 mesh, BIO-RAD) to absorb KCN. The organic layer was dried over $Na_2SO_4$, followed by vacuum evaporation to give the desired product (0.64 g, 98%, **14**—77%, **15**—23%, $R_f$(**14**)=0.16, $R_f$ (**15**)=0.22 in 1/19 $CH_3OH/CH_2Cl_2$); $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 8.86 (s, 1H, NH, **14**), 8.78 (s, 1H, NH, **15**), 7.32–7.10 (m, 8H, aromatic, **14** and **15**), 4.51 (s, 2H, C$H_2$Ph, **14**), 4.48 (s, 2H, C$H_2$Ph, **15**), 2.68 (br s, 2H, $NH_2$, **14**), 2.54 (br s, 2H, $NH_2$, **15**), 2.01–1.68 (m, 16H, central ring, **14** and **15**); $^{13}$C NMR (75.5 MHz, DMSO-$d_6$): $\delta$ 176.1 (C1′), 163.0 and 159.8 (d, C–F, *J*=243 Hz), 155.4 (C3′ of **14**), 155.3 (C3′ of **15**), 133.0 and 132.9 (aromatic), 129.3 and 129.2 (aromatic of **14**), 128.4 and 128.3 (aromatic of **15**), 125.2 (CN of **15**), 124.0 (CN of **14**), 115.9 and 115.2 (aromatic of **14**, *J*=21.0 Hz), 114.9 and 114.6 (aromatic of **15**, *J*=20.9 Hz), 62.1 (C1 of **15**), 59.7 (C1 of **14**), 50.2 (C4 of **14**), 47.9 (C4 of **15**), 40.3 (*C*$H_2$Ph), 32.2 (C2 and C6 of **14**), 31.0 (C2 and C6 of **15**), 30.1 (C3 and C5 of **14**), 27.8 (C3 and C5 of **15**); HRMS-CI calcd for $C_{16}H_{18}N_4O_2F$ $[M+H]^+$: 317.1414, found 317.1415.

**4.1.12. *trans*-3-(4-Fluoro-benzyl)-1,3,9,11-tetraaza-dispiro[4.2.4.2]tetradecane-2,4,10,12-tetraone (16 and 17).** To a mixture of **14** and **15** (100 mg, 0.316 mmol) in 4 mL of $CH_2Cl_2$ was added chlorosulfonyl isocyanate (48 mg, 0.34 mmol). After being stirred for 10 min at room temperature, the clear solution was concentrated in vacuo into a pale yellow form. Following addition of 3 mL of 1 N HCl the suspension was stirred for 10 min at room temperature, then heated in an oil bath at 100 °C for 1 h. After approximately 15 min of heating, the reaction mixture became homogeneous, and then a precipitate formed. The reaction mixture was cooled to room temperature; the solid was filtered, washed with water, and dried to give 0.08 g of a mixture (70%) of **16** and **17** (7/3): $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 10.63 (s, 1H, NH, **17**), 10.60 (s, 1H, NH, **16**), 8.98 (s, 1H, NH, **17**), 8.66 (s, 1H, NH, **16**), 8.61 (s, 1H, NH, **17**), 8.25 (s, 1H, NH, **16**), 7.28–7.24 (m, aromatic, **16** and **17**), 7.17–7.13 (m, aromatic, **16** and **17**), 4.50 (s, 2H, C$H_2$Ph, **17**), 4.49 (s, 2H, C$H_2$Ph, **16**), 2.12–2.07 (m, 4H, central ring, **16**), 2.00–1.97 (m, 4H, central ring, **17**), 1.70–1.60 (m, 8H, central ring, **16** and **17**); $^{13}$C NMR (75.5 MHz, DMSO-$d_6$): $\delta$ 178.0 (C1′ of **16**), 177.8 (C1′ of **17**), 176.1 (C1″ of **16**), 176.0 (C1″ of **17**), 161.0 (d, C–F, $J_{CF}$=239 Hz, **16** and **17**), 156.4 (C3′ of **16** and **17**), 155.6 (C3″ of **16**), 155.5 (C3″ of **17**), 133.0 (aromatic, **16** and **17**), 129.2 (d, *C*–(C–C–F), $^3J_{CF}$=8 Hz, **16**), 129.1 (d, *C*–(C–C–F), $^3J_{CF}$=8 Hz, **17**), 115.3 (d, *C*–(C–F), $^2J_{CF}$=21.9 Hz, **16** and **17**), 60.8 (C1 of central ring of **17**), 60.0 (C4 of central ring of **17**), 59.6 (C1 of central ring of **16**), 58.9 (C4 of central ring of **16**), 40.2 (*C*$H_2$Ph of **17**), 40.1 (*C*$H_2$Ph of **16**), 29.1 (C2 and C6 of central ring of **16**), 29.0 (C3 and C5 of central ring of **16**), 28.6 (C2 and C6 of central ring of **17**), 28.5 (C3 and C5 of central ring of **17**); HRMS-CI calcd for $C_{17}H_{18}N_4O_4F$ $[M+H]^+$: 361.1312, found 361.1297.

**4.1.13. (4-Hydroxymethyl-cyclohexa-1,3-dienyl)-methanol (19).** Starting material **18** (1.8 g, 9.25 mmol) was dissolved in anhydrous THF (20 mL) at 0 °C, which was then subject to ultra-pure Ar flushing for 10 min. DIBAL-H (37 mL) in THF (1.0 M, 37 mmol) was added to the above solution slowly via a double-ended needle. The clear solution was stirred rigorously at 0 °C for 30 min then quenched with MeOH (3 mL), followed by adding powdered $Na_2SO_4\cdot10H_2O$ (25 g). The mixture was stirred at room temperature for 1 h and then filtered through Celite, and the filter cake was washed by copious EtOAc. The solution was evaporated in vacuo yielding a light orange oil. The oil was

dissolved in EtOAc and applied to a flash column using a $CH_2Cl_2$/EtOAc gradient to elute the product (1.14 g, 88%, $R_f$=0.24 in 3/2 $CH_2Cl_2$/EtOAc). Due to the instability of the product, it was used immediately for the next step: $^1$H NMR (300 MHz, $CDCl_3$): $\delta_H$ 5.89 (s, 2H, CH–CH), 4.15 (d, 4H, 2C$H_2$OH, $J$=5.7 Hz), 2.22 (s, 4H, 2$CH_2$), 1.93 (t, 2H, 2OH, $J$=5.7 Hz); $^{13}$C NMR (75.5 MHz, $CDCl_3$): $\delta_C$ 137.6 (C1 and C4), 119.0 (C2 and C3), 66.0 ($CH_2$OH), 23.9 (C5 and C6); HRMS-CI calcd for $C_8H_{12}O_2$ [$M^+$]: 140.0837, found 140.0839.

**4.1.14. 1,4-Bis-hydroxymethyl-2,3-diaza-bicyclo [2.2.2]oct-5-ene-2,3-dicarboxylic acid diethyl ester (20).** Diethyl azodicarboxylate (DEAD) (5.0 mL, 11.0 mmol) was added to a $CH_2Cl_2$ (40 mL) solution of **19** (1.40 g, 10 mmol). The solution was stirred at room temperature for 24 h. The adduct **20** was isolated by column chromatography using a $CH_2Cl_2$/EtOAc gradient to give a viscous, colorless oil (1.13 g, 36%, $R_f$=0.32 in 1/1 $CH_2Cl_2$/EtOAc): $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 6.86 (d, 1H, C$H$=CH, $J$=8.1 Hz), 6.26 (d, 1H, CH=C$H$, $J$=8.1 Hz), 4.27–4.04 (m, 8H, 2C$H_2$OH and 2OC$H_2$CH$_3$), 2.39–2.32 (m, 1H, C$H$HCH$_2$), 1.98–1.91 (m, 1H, CH$H$CH$_2$), 1.45–1.37 (m, 1H, CH$_2$CH$H$), 1.29–1.19 (m, 7H, CH$_2$C$H$H and 2CH$_3$); $^{13}$C NMR (75.5 MHz, $CDCl_3$): $\delta$ 159.3 ($C$O$_2$Et), 158.1 ($C$O$_2$Et), 136.5 ($C$=C), 135.5 (C=$C$), 66.4, 65.9, 65.2, 63.2, 62.8, 62.5, 27.5 ($C$H$_2$CH$_2$), 26.7 (CH$_2$$C$H$_2$), 14.5 (CH$_3$), 14.3 (CH$_3$); HRMS-CI calcd for $C_{14}H_{23}N_2O_6$ [M+H]$^+$: 315.1556, found 315.1543.

**4.1.15. 1,4-Bis-methoxymethoxymethyl-2,3-diaza-bicyclo[2.2.2]oct-5-ene-2,3-dicarboxylic acid diethyl ester (21).** A solution of **20** (1.0 g, 3.1 mmol), MOM-Cl (0.5 mL, 6.25 mmol), DIPEA (1.40 mL, 7.75 mmol) in dry $CH_2Cl_2$ (40 mL) under $N_2$ was stirred at room temperature for 24 h, then quenched with 40 mL saturated $NaHCO_3$ solution. The layers were separated, and the aqueous layer was extracted with $CH_2Cl_2$. The combined organic layers were washed with water, brine, dried ($Na_2SO_4$), filtered, and concentrated in vacuo. Flash chromatography on silica gel ($CH_2Cl_2$/EtOAc, 3/2) gave **21** as a colorless oil (1.18 g, 95%); $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 6.65 (d, 1H, C$H$=CH, $J$=8.0 Hz), 6.51 (d, 1H, CH=C$H$, $J$=8.0 Hz), 4.74–4.72 (m, 4H, 2$CH_2$), 4.54–4.49 (m, 2H, $CH_2$), 4.26–4.03 (m, 6H, 3$CH_2$), 3.39 (s, 6H, 2$CH_3$), 2.34–3.30 (m, 1H, C$H$HCH$_2$), 1.88–1.81 (m, 2H, C$H_2$CH$_2$), 1.35–1.28 (m, 1H, CH$H$CH$_2$), 1.26 (t, 3H, $CH_3$, $J$=6.8 Hz), 1.19 (t, 3H, $CH_3$, $J$=7.2 Hz); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ 158.9 ($C$O$_2$Et), 157.7 ($C$O$_2$Et), 137.7 ($C$=C)), 134.8 (C=$C$), 96.6 (OCH$_2$O), 96.4 (OCH$_2$O), 69.8, 69.3, 62.8, 61.9, 61.6, 61.2, 55.2, 55.1, 28.1 ($C$H$_2$CH$_2$), 25.0 (CH$_2$$C$H$_2$), 14.3 ($C$H$_3$CH$_2$), 14.2 ($C$H$_3$CH$_2$); HRMS-CI calcd for $C_{18}H_{31}N_2O_8$ [M+H]$^+$: 403.2080, found 403.2082.

**4.1.16. *cis*-(4-Ethoxycarbonylamino-1,4-bis-methoxymethoxymethyl-cyclohexyl)-carbamic acid ethyl ester (22).** Under dry Ar, 10% Pd/C (0.15 g) catalyst was added slowly into a solution of **21** (0.55 g, 1.37 mmol) in $CH_2Cl_2$ (30 mL). The solution was degassed, placed under hydrogen atmosphere (10 psi), and stirred for 24 h. The reaction mixture was filtered through Celite on a fine fritted glass filter and the activated carbon was washed with additional EtOAc. The filtrate was evaporated in vacuo to dryness then redissolved in THF (40 mL). The THF solution was then transferred to a three-neck flask into which $NH_3$ was bubbled at −70 °C until approximately 150 mL were condensed. Excess Na metal was added and the solution turned dark blue. The resulting mixture was stirred at −70 °C for 1 h. The reaction was quenched by carefully adding solid $NH_4Cl$, and $NH_3$ was allowed to evaporate slowly. The residue was diluted with EtOAc (150 mL) and filtered, and the solvents were evaporated in vacuo. The resulting crude product was purified by flash chromatography over silica gel ($CH_2Cl_2$/EtOAc 5/1) to give **22** as a colorless paste (0.52 g, 95%); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 4.70 (s, 2H, 2NHCO), 4.58 (s, 4H, 2$CH_2$), 4.03 (q, 4H, 2$CH_2$, $J$=7.2 Hz), 3.66 (s, 4H, 2$CH_2$), 3.32 (s, 6H, 2$CH_3$), 2.02–1.94 (m, 4H, equatorial), 1.70–1.64 (m, 4H, axial), 1.20 (t, 6H, 2$CH_3$, $J$=7.2 Hz); $^{13}$C NMR (75.5 MHz, $CDCl_3$): $\delta$ 155.1 ($C$O$_2$C$_2$H$_5$), 96.6 (OCH$_2$O), 69.4 (O$C$H$_2$C), 60.2 (O$C$H$_2$CH$_3$), 55.2 (OCH$_3$), 54.3 (C1 and C4 of the ring), 27.3 (C2, C3, C5, and C6 of the ring), 14.5 ($C$H$_3$CH$_2$); HRMS-CI calcd for $C_{18}H_{35}N_2O_8$ [M+H]$^+$: 407.2393, found 407.2388.

**4.1.17. *cis*-(4-*tert*-Butoxycarbonylamino-1,4-bis-hydroxymethyl-cyclohexyl)-carbamic acid *tert*-butyl ester (23).** Iodotrimethyl silane (TMSI, 0.58 mL, 4.3 mmol) was added dropwise into a solution of **22** (174 mg, 0.43 mmol) in $CH_2Cl_2$ (20 mL). The reaction mixture was heated under reflux for 18 h and quenched with MeOH (1.0 mL), leading to the formation of a fine precipitates. Vacuum filtration gave a yellowish solid (presumably an iodide salt of the fully deprotected dihydroxyl diamine). This highly hygroscopic solid was used directly in the next step without further purification. MeOH (10 mL), di-*tert*-butyl-dicarbonate (0.25 g, 1.14 mmol), and triethyl amine (0.24 mL, 1.71 mmol) were added to the above solid and the reaction mixture was stirred at room temperature overnight, then quenched with 40 mL saturated $NaHCO_3$ solution. The layers were separated and the aqueous layer was extracted with EtOAc (2×40 mL). The combined organic layers were washed with water, brine, dried ($Na_2SO_4$), filtered, and concentrated in vacuo. Flash chromatograph on silica gel ($CH_2Cl_2$/MeOH 9/1) gave **23** as a colorless solid (119 mg, 74%); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 4.61 (s, 2H, 2NHCO), 3.70 (s, 4H, 2C$H_2$OH), 1.72 (s, 8H, equatorial and axial), 1.44 (s, 18H, 2C(CH$_3$)$_3$); $^{13}$C NMR (75.5 MHz, $CDCl_3$): $\delta$ 156.2 ($C$O$_2$C(CH$_3$)$_3$), 80.2 (CH$_2$OH), 68.0 ($C$(CH$_3$)$_3$), 55.6 (C1 and C4), 28.3 (CH$_3$), 27.8 (C2, C3, C5, and C6); HRMS-CI calcd for $C_{18}H_{35}N_2O_6$ [M+H]$^+$: 375.2495, found 375.2498.

**4.1.18. *cis*-1,4-Bis-*tert*-butoxycarbonylamino-cyclohexane-1,4-dicarboxylic acid (24).** A solution of bis-alcohol **23** in *tert*-butyl alcohol (1 mL/0.1 mmol of alcohol) was treated at room temperature with sodium hydroxide solution (4 equiv, 0.5 M in water) and $KMnO_4$ solution (4 equiv, 10% water) and the resulting mixture was stirred overnight. After quenching the reaction with an excess of aqueous sodium thiosulfate (5%), the mixture was washed with diethyl ether and the aqueous solution was acidified to a pH of 1–2 with 1 M HCl at 0 °C. The mixture was extracted with ethyl acetate three times and the combined organic layers were dried ($Na_2SO_4$), filtered, and concentrated in vacuo to yield product **24** as a colorless, sticky solid that was used for the next

step without further purification (93%); $^1$H NMR (300 MHz, $CD_3OD$): $\delta$ 2.19–2.15 (m, 4H, equatorial), 1.89–1.84 (m, 4H, axial), 1.42 (s, 18H, $2C(CH_3)_3$); $^{13}$C NMR (75.5 MHz, $CDCl_3$): $\delta$ 177.6 ($CO_2H$), 157.3 (CONH), 80.1 ($C(CH_3)_3$), 58.9 (C1 and C4), 30.1 (C2, C3, C5, and C6), 28.7 ($CH_3$); HRMS-CI calcd for $C_{18}H_{31}N_2O_8$ $[M+H]^+$: 403.2080, found 403.2075.

**4.1.19. *cis*-1,4-bis-*tert*-Butoxycarbonylamino-cyclohexane-1,4-dicarboxylic acid dimethyl ester (25).** Ester **25** was prepared from **24** by following the procedure described for the synthesis of **5** in 95% yield; $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 4.83 (s, 2H, 2NH), 3.73 (s, 6H, $2CH_3$), 2.26–2.21 (m, 4H, equatorial), 1.88–1.82 (m, 4H, axial), 1.43 (s, 18H, $2C(CH_3)_3$); $^{13}$C NMR (75.5 MHz, $CDCl_3$): $\delta$ 173.9 ($CO_2CH_3$), 154.8 (CONH), 80.1 ($C(CH_3)_3$), 57.7 (C1 and C4), 52.3 ($OCH_3$), 31.4 (C2, C3, C5, and C6), 28.2 ($CH_3$); HRMS-CI calcd for $C_{20}H_{35}N_2O_8$ $[M+H]^+$: 431.2393, found 431.2378.

**4.1.20. *cis*-1,4-Bis-((*N*-benzoyl(thiocarbamoyl)amino)-1,4-dimethoxycarbonyl-cyclohexane (27).** The ester **25** (0.7 g, 1.63 mmol) was treated with 35 mL $CH_2Cl_2$/TFA (3/7) at 0 °C. The reaction mixture was stirred at room temperature for 4 h. The volatiles were removed under vacuum and the residue was kept in a dry ice/acetone cold bath, to which a diluted TEA (5 mL) in $CH_2Cl_2$ (10 mL) solution was added slowly (caution: fumes formed). After 10 min of stirring, the cold bath was removed and the reaction mixture was warmed to room temperature. The volatiles were all removed in vacuo, giving **26** as a colorless oil, which was redissolved in dry $CH_2Cl_2$ (15 mL). Benzoyl isothiocyanate (0.48 mL, 3.58 mmol) was added by syringe. The clear solution was stirred for 12 h and then concentrated in vacuo. Flash chromatograph on silica gel (9/1 $CH_2Cl_2$/EtOAc) gave **27** as a yellowish oil (0.70 g, 77%); $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 11.15 (s, 2H, 2NHCO), 8.93 (s, 2H, 2NHC=S), 7.84–7.82 (m, 4H, aromatic), 7.63–7.61 (m, 2H, aromatic), 7.54–7.50 (m, 4H, aromatic), 3.79 (s, 6H, $2CH_3$), 2.70–2.65 (m, 4H, equatorial), 2.27–2.22 (m, 4H, axial); $^{13}$C NMR (75.5 MHz, $CDCl_3$): $\delta$ 180.8 (C=S), 173.0 ($CO_2Me$), 168.0 (CONH), 134.3, 132.9, 128.8 and 128.6 (aromatic), 70.2 ($CH_2C_6H_5$), 60.2 (C1 and C4), 52.5 ($CH_3$), 26.1 (C2, C3, C5, and C6); HRMS-CI calcd for $C_{26}H_{29}N_4O_6S_2$ $[M+H]^+$: 557.1529, found 557.1544.

**4.1.21. *cis*-2,10-Dithioxo-1,3,9,11-tetraaza-dispiro [4.2.4.2]tetradecane-4,12-dione (28).** A mixture of **27** (0.55 g, 0.99 mmol) and triethyl amine (1.5 mL) in methanol (10 mL) was heated at reflux for 18 h. The solvent was evaporated; ethanol (10 mL) was added to the residue and the solution was adjusted to a pH of 5.0 with a 5% aqueous HCl. The precipitate was collected by filtration to give **28** as white solid (0.21 g, 75%); $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 11.7 (br s, 2H, 2NH(C=O)(C=S)), 10.4 (s, 2H, 2NHC=S), 2.09–2.05 (m, 4H, equatorial), 1.79–1.74 (m, 4H, axial); $^{13}$C NMR (75.5 MHz, DMSO-$d_6$): $\delta$ 182.1 (C=S), 178.2 (C=O), 63.5 (C1 and C4), 27.9 (C2, C3, C5, and C6); HRMS-CI calcd for $C_{10}H_{13}N_4O_2S_2$ $[M+H]^+$: 285.0480, found 285.0478.

**4.1.22. *cis*-2,10-Dihydrazino-1,3,9,11-tetraaza-dispiro [4.2.4.2]tetradeca-1,9-diene-4,12-dione (30).** Compound **30** was prepared from **28** by following the procedure described for the synthesis of **10** in 72% overall yield in two steps; $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 9.03 (br s, 2H, 2NH), 7.29 (s, 2H, $2NH(NH_2)$), 4.46 (s, 4H, $2NH_2$), 1.97–1.93 (m, 4H, equatorial), 1.62–1.57 (m, 4H, axial); $^{13}$C NMR (75.5 MHz, DMSO-$d_6$+TFA): $\delta$ 177.8 (C=O), 158.6 ($NH_2$–C=N), 64.3 (C1 and C4), 29.0 (C2, C3, C5, and C6); HRMS-CI calcd for $C_{10}H_{17}N_8O_2$ $[M+H]^+$: 281.1474, found 281.1482.

**4.1.23. Bis-intercalator D1.** The linker **10** (0.05 mg, 0.18 mmol) was added into a DMF solution (15 mL) containing naphthalenetetracarboxylic diimide **31** (0.26 g, 0.54 mmol),[17] PyBOP (0.279 g, 0.54 mmol), HOBT (0.082 g, 0.54 mmol), and DIPEA (0.19 mL, 1.1 mmol). The reaction mixture was stirred at room temperature for 20 h then filtered, and the crude solid product was rinsed with DMF (2×10 mL) and then with ethanol (2×10 mL). The solid was dried in vacuo, then redissolved in 1/1 TFA/$CH_2Cl_2$ (20 mL). The solvent was removed under vacuum after 15 min of stirring. The resulting pink solid was dissolved in 0.1% TFA/$H_2O$ and purified by reverse-phase preparative HPLC (Amersham, AKTA Purifier, Hi-Pore RP-318 250×10 mm column) using 0.1% TFA/water as solvent A and 0.1% TFA/ACN as solvent B. The product fractions were combined and lyophilized to yield the product **D1** (0.12 g, 70%) as a light orange solid; $^1$H NMR (500 MHz, $D_2O$): $\delta$ 8.60–8.56 (m, 8H), 4.68 (br s, 4H), 4.41 (t, 4H, $J$=6.5 Hz), 3.35 (br s, 4H), 2.75 (t, 4H, $J$=6.5 Hz), 1.52 (br s, 8H); UV–vis: $\lambda_{max}$ ($\log_{10}\varepsilon$) 383 (4.56), 363 nm (4.46); MALDI (Applied Biosystems 4700 Proteomics Analyzer, Foster City, CA) calcd for $C_{48}H_{43}N_{14}O_{12}$ $[M+H]^+$: 1007, found 1007.

**4.1.24. Bis-intercalator C1.** The linker **30** (20.5 mg, 0.073 mmol) was added into a DMF solution containing naphthalenetetracarboxylic diimide **31** (106 mg, 0.22 mmol),[17] PyBOP (114 mg, 0.22 mmol), HOBT (34 mg, 0.22 mmol), and DIPEA (0.08 mL, 0.44 mmol). The reaction mixture was stirred for 20 h at room temperature. Diethyl ether (20 mL) was added to the above clear solution at −20 °C and a precipitate formed. Vacuum filtration gave a light orange solid that was then dissolved in 1/1 TFA/$CH_2Cl_2$ (20 mL). The solvents were removed under vacuum after 15 min of stirring at room temperature. The resulting solid was dissolved in 0.1% aqueous TFA and purified following the same procedure as for **D1**. The product fractions were combined and lyophilized to yield the product **C1** (44 mg, 60%) as a light orange solid; $^1$H NMR (500 MHz, $D_2O$): $\delta$ 8.41 (d, 4H, aromatic Hs, $J$=6.0 Hz), 8.30 (d, 4H, aromatic Hs, $J$=6.0 Hz), 4.68 (br s, 4H), 4.43 (t, 4H, $J$=6.1 Hz), 3.35 (t, 4H, $J$=6.1 Hz), 2.65 (br s, 4H), 2.29 (m, 4H), 1.97 (m, 4H); UV–vis: $\lambda_{max}$ ($\log_{10}\varepsilon$) 384 (4.42), 364 nm (4.43); MALDI (Applied Biosystems 4700 Proteomics Analyzer, Foster City, CA) calcd for $C_{48}H_{43}N_{14}O_{12}$ $[M+H]^+$: 1007, found 1007.

### 4.2. Dissociation kinetics

In a typical experiment, 500 μL of 0.5 mM of DNA stock solution in PIPES buffer (containing 50 mM NaCl, pH 7.0) was first incubated with 500 μL of 0.04 mM compound stock solution for 2 h. Next, 0.5 mL of the resulting

DNA/compound complex solution was mixed with 0.5 mL of 4% SDS solution in a quartz cuvette with 1 cm path length. The UV absorbance was monitored at 383–385 nm (depending on the linker structure of the dimer) after 20 s of mixing (Hewlet-Packard 8553 Multitransport UV–vis spectrophotometer).

### 4.3. Computer simulation

Molecular dynamics and geometry optimization computations were performed in HyperChem 7.0 (Hypercube Inc., 1115 NW 4th Street, Gainesville, FL 32601) using the Amber force field. PDB coordinates from the **G$_3$K**/d $(CGGTACC)_2$ were used and the linker segment was modified as needed.[19] During the calculation, DNA coordinates were fixed and molecular dynamics were performed on the dimer to anneal the system to obtain a lower energy minimum. Initial structures were subjected to 15 picoseconds of molecular dynamics at 1000 K to allow high degree randomization of the initial model, and then the temperature was slowly lowered to 300 K over 10 ps. After annealing a final geometry optimization was performed using the Fletcher–Reeves conjugate gradient algorithm, with a convergence cutoff value of 0.01 kcal mol$^{-1}$.

### 4.4. X-ray single crystallographic analysis

Crystallographic summary for **5** ($C_{26}H_{30}N_2O_8$): colorless prismatic crystals, monoclinic, $P2_1/c$ (No. 14), $Z$=2 in a cell of dimensions: $a$=12.7888(9), $b$=10.5763(7), $c$=9.7425(5) Å, $\beta$=105.064(4)°, $V$=1272.47(14) Å$^3$, $\rho_{calc}$=1.30 g cm$^{-3}$, $\mu$=0.097 mm$^{-1}$, $F(000)$=528. A total of 4024 reflections were measured, 2200 unique ($R_{int}$=0.056), on a Nonus Kappa CCD using graphite monochromatized Mo K$\alpha$ radiation ($\lambda$=0.71073 Å) at −120 °C. The structure was refined on $F^2$ to an $R_w$=0.254, with a conventional $R$=0.100 (1451 reflections with $F_o>4[\sigma(F_o)]$), and a goodness of fit=1.489 for 198 refined parameters.

Crystallographic summary for **8** ($C_{10}H_{12}N_4O_2S_2$–$2C_2H_6SO$: colorless prismatic crystals, monoclinic, $P2_1/n$ (No. 14), $Z$=2 in a cell of dimensions: $a$=8.0735(1), $b$=9.2155(2), $c$=13.6410(3) Å, $\beta$=99.819(1)°, $V$=1000.04(3) Å$^3$, $\rho_{calc}$=1.46 g cm$^{-3}$, $\mu$=0.502 mm$^{-1}$, $F(000)$=464. A total of 4213 reflections were measured, 2275 unique ($R_{int}$=0.028), on a Nonus Kappa CCD using graphite monochromatized Mo K$\alpha$ radiation ($\lambda$=0.71073 Å) at −120 °C. The structure was refined on $F^2$ to an $R_w$=0.0769, with a conventional $R$=0.0349 (1765 reflections with $F_o>4[\sigma(F_o)]$), and a goodness of fit=1.010 for 167 refined parameters.

Crystallographic summary for **12a** (an analogue of **12**) ($C_{17}H_{20}N_2O_4$): colorless needles, triclinic, $P$-1 (No. 2), $Z$=2 in a cell of dimensions: $a$=5.6474(2), $b$=11.2341(4), $c$=12.4607(4) Å, $\alpha$=99.586(2), $\beta$=97.391(2), $\gamma$=91.882(2)°, $V$=771.84(5) Å$^3$, $\rho_{calc}$=1.36 g cm$^{-3}$, $\mu$=0.098 mm$^{-1}$, $F(000)$=336. A total of 5249 reflections were measured, 3497 unique ($R_{int}$=0.026), on a Nonus Kappa CCD using graphite monochromatized Mo K$\alpha$ radiation ($\lambda$=0.71073 Å) at −120 °C. The structure was refined on $F^2$ to an $R_w$=0.108, with a conventional $R$=0.0431 (2188 reflections with $F_o>4[\sigma(F_o)]$), and a goodness of fit=1.006 for 289 refined parameters.

Crystallographic summary for **28** ($(C_{10}H_{11}N_4S_2O_2)^{1-}$ $(C_6H_{16}N)^{1+}$): colorless lathes, monoclinic, $P2_1/c$ (No. 14), $Z$=8 in a cell of dimensions: $a$=12.2470(3), $b$=11.9535(3), $c$=27.0891(7) Å, $\beta$=96.674(1)°, $V$=3938.82(17) Å$^3$, $\rho_{calc}$=1.30 g cm$^{-3}$, $\mu$=0.290 mm$^{-1}$, $F(000)$=1648. A total of 11,100 reflections were measured, 6658 unique ($R_{int}$=0.070), on a Nonus Kappa CCD using graphite monochromatized Mo K$\alpha$ radiation ($\lambda$=0.71073 Å) at −120 °C. The structure was refined on $F^2$ to an $R_w$=0.140, with a conventional $R$=0.0617 (3580 reflections with $F_o>4[\sigma(F_o)]$), and a goodness of fit=1.168 for 460 refined parameters.

Crystallographic data (excluding structure factors) for **5**, **8**, **12a**, and **28** reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication numbers CCDC 293250, 293251, 293248, and 293249, respectively. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax: +44 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk].

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## References and notes

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