ACS Medicinal Chemistry Letters

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DNA Sequence Preference and Adduct Orientation of Pyrrolo[2,1-*c*][1,4]benzodiazepine Antitumor Agents

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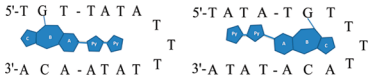
ABSTRACT The pyrrolobenzodiazepines (PBDs) are covalent DNA minor-groove binding agents with a reported preference for binding to 5′-Pu-G-Pu sequences with their A rings oriented toward the 3′-end of the covalently modified DNA strand. Using HPLC/MS methodology and a range of designed hairpin-forming 17-mer oligonucleotides, the kinetics of reaction of a *bis*-pyrrole PBD conjugate (GWL-78, **2**) has been evaluated with eight isomeric oligonucleotides, each containing a single PBD binding site in one of two locations. The PBD-binding base pair triplets were designed to include every possible combination of A and T bases adjacent to the covalently reacting guanine. Contrary to expectations, **2** reacted most rapidly with TGT and TGA sequences, and adducts were observed to form in both the 3′- and the 5′-directions. Molecular modeling studies revealed that for 3′-oriented adducts, this preference could be explained by formation of a hydrogen bond between the N10-H of the PBD and the oxygen of the C2-carbonyl of a thymine base on the 3′-side of the covalently bound guanine. For 5′-adducts, an analogous PBD N10-H hydrogen bond may form instead to the N3 of an equivalent adenine on the opposite strand.

KEYWORDS Pyrrolo[2,1-*c*][1,4]benzodiazepine (PBD), antitumor agents, GWL-78, DNA, minor-groove binder



The pyrrolo[2,1-*c*][1,4]benzodiazepines (PBDs) are a well-known class of sequence-selective covalent-binding DNA-interactive agents[1−4] that fit perfectly in the minor groove of DNA due to their chiral C11a(*S*)-position, which provides a right-handed longitudinal twist isohelical with double-stranded DNA.[3] They also possess an electrophilic N10−C11 imine moiety (or the carbinolamine or carbinolamine methyl ether equivalent) that can form a covalent aminal linkage between their C11-position and the nucleophilic C2-NH_2 group of a guanine base.[3] The simple monomeric PBDs such as the natural products anthramycin (Figure 1) and tomaymycin typically span three base pairs of DNA. There are numerous reports in the literature based on NMR,[5] X-ray crystallography,[6] molecular modeling,[7] and gel-based experiments (e.g., DNA footprinting[8] and in vitro polymerase stop assays[9]) suggesting that they have a rank order of preference for binding to 5′-Pu-G-Pu-3′ > Pu-G-Py ∼ Py-G-Pu > Py-G-Py sequences with the adducts oriented so that the PBD A ring points toward the 3′ terminus of the covalently modified strand. The PBDs have been shown to mediate a number of biological effects including the inhibition of endonuclease[10] and RNA polymerase[9,11] enzymes, and inhibition of binding of the transcription factor NF**Y**.[12]

The covalent binding of PBDs to DNA is presumed to be a two-step process,[11] the first involving recognition of a favored low-energy binding site by fast, reversible noncovalent association of the drug in the minor groove through interactions including hydrogen-bonding, van der Waals, and electrostatic contacts. If these noncovalent intermolecular interactions are weak, the molecule presumably dissociates and then reassociates at another site, with the process repeating itself until the PBD finds a suitable low-energy triplet with the C2-NH_2 of a central guanine aligned for nucleophilic attack at the PBD C11-position. The second step should then occur, with covalent bond formation between the guanine and the PBD, at which point the molecule will be locked into position. The rate of this second covalent step is much slower than the initial noncovalent association and can, according to the literature, take up to 24 h to complete.

GWL-78[11] (**2**, Figure 1) is a PBD-C8-dipyrrole conjugate designed to span five DNA base pairs with two AT base pairs (recognized by the *bis*-pyrrole rings) adjacent to a triplet PBD binding site. It has been shown to successfully penetrate cell and nuclear membranes to reach the nucleus where it interacts covalently with DNA and interferes with cell cycle progression.[12,13] More specifically, **2** has been shown to inhibit binding of the NF**Y** transcription factor to its cognate sequences (e.g., CCAAT) within the topoisomerase IIα promoter region, thus causing a decrease in topo IIα expression and blocking cell cycle progression, ultimately leading to apoptosis.[12]

Received Date: June 7, 2010
Accepted Date: July 25, 2010
Published on Web Date: August 13, 2010

DOI: 10.1021/ml100131b | *ACS Med. Chem. Lett.* **2010**, *1*, 427–432

ACS Medicinal Chemistry Letters
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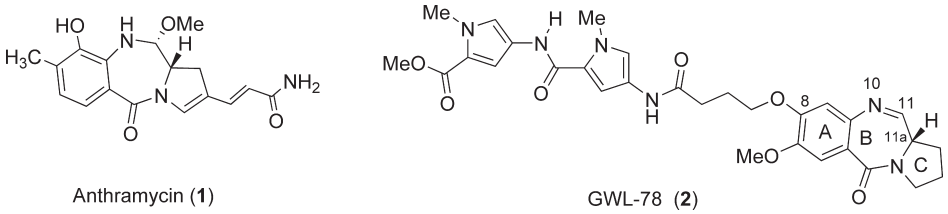

Figure 1. Naturally occurring PBD monomer anthramycin methyl ether (**1**) and the *bis*-pyrrole PBD C8-conjugate **2** (GWL-78).

Table 1. Rate Constants (μM/min) and Free Energy Changes (ΔG) for Covalent Reaction of the *bis*-Pyrrole PBD C8-Conjugate **2** (GWL-78) with the Isomeric Oligonucleotides Seq-1 to Seq-8[a]

label	hairpin sequence	A ring orientation of adducts	rate constant (k') for reaction with **2** (μM/min)	change in free energy upon noncovalent interaction (ΔG, kcal/mol)
	binding site located next to the loop region:			
Seq-1	5′-TATA-AGA-TTT-TCT-TATA	A ring 5′	0.091 ± 0.005	−50.15
Seq-2	5′-TATA-TGT-TTT-ACA-TATA	A ring 5′	$\geq 0.506 \pm 0.012$	−48.39
Seq-3	5′-TATA-AGT-TTT-ACT-TATA	A ring 5′	0.124 ± 0.007	−48.20
Seq-4	5′-TATA-TGA-TTT-TCA-TATA	A ring 5′	$\geq 0.364 \pm 0.012$	−48.94
	binding site located at the 5′ terminus:			
Seq-5	5′-AGA-TATA-TTT-TATA-TCT	A ring 3′	0.132 ± 0.006	−50.37
Seq-6	5′-TGT-TATA-TTT-TATA-ACA	A ring 3′	$\geq 0.808 \pm 0.008$	−52.87
Seq-7	5′-AGT-TATA-TTT-TATA-ACT	A ring 3′	0.096 ± 0.003	−51.98
Seq-8	5′-TGA-TATA-TTT-TATA-TCA	A ring 3′	0.196 ± 0.004	−52.79

[a] Based on structural and energetic considerations, adducts with Seq-1 to Seq-4 are expected to form with the PBD A ring oriented towards the 5′ terminus of the oligonucleotide, whereas adducts with Seq-5 to Seq-8 should be orientated 3′.

A high-performance liquid chromatography/mass spectrometry (HPLC/MS) assay developed in our laboratory[14−16] has been used to study the reaction of **2** with a set of eight hairpin oligonucleotides (Table 1) to establish both the rate of alkylation and the orientation of the PBD molecule in the adducts formed. Each oligonucleotide contained a single PBD-binding triplet at one of two different locations, with the triplets containing all possible combinations of X-G-X, where X was either A or T. The use of G or C (for X) was avoided to limit the number of possible sites of covalent interaction to one per oligonucleotide. The X-G-X triplets were positioned in one of two possible locations to allow investigation of the binding orientation of the PBD molecule. In the first four oligonucleotides (Seq-1 to Seq-4, Table 1), the PBD binding site was situated immediately adjacent to the 5′-end of the central TTT loop. The hairpin sequences, which have no direct biological relevance, were designed to be of adequate length (i.e., 17-mer) to ensure formation of a minor groove environment within the hairpin structure, as we have previously shown (unpublished results) that a minimum of seven base pairs are required to provide sufficient minor groove environment for covalent attachment of a PBD. The 17-mer sequences were thus the minimum possible length accounting for the three-base TTT loop region and seven base pairs on each arm of the hairpin. For these oligonucleotides, **2** was expected to bind with its C8-*bis*-pyrrole component oriented to the 5′-end of the oligonucleotide so that the pyrrole units could be accommodated in the minor groove environment of the stem region (Figure 2A). In this case, the opposite 3′-orientation would be unlikely as, according to molecular modeling studies, the *bis*-pyrrole component would have to protrude through the −TTT− loop of the hairpin entailing significant steric clashes. In the second set of four oligonucleotides (Seq-5 to Seq-8, Table 1), the binding site was positioned at the 5′ terminus of the stem region in which case **2** should orientate with its A ring toward the 3′-end so that, once again, the pyrrole units can be accommodated within the minor groove environment of the stem (Figure 2B). In this case, the opposite orientation was also considered unlikely, as the *bis*-pyrrole component would then have to protrude beyond the 5′-end of the hairpin with a loss of DNA/ligand stabilizing interactions and the possibility that solvation forces may destabilize the adduct.

Interaction of **2** with oligonucleotides Seq-1 to Seq-8 was studied using an HPLC assay and the **2**/oligonucleotide adducts were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF-MS). All eight oligonucleotides are isomeric with a theoretical mass of 5173.49 each. Covalent attachment of one molecule of **2** to any oligonucleotide should increase the mass to a theoretical value of 5764.12. Values ranging from 5761.63 to 5787.90 were observed for the eight adducts formed, all within experimental margin of error. It is known from previous studies that only covalent adducts provide MALDI TOF signals, as noncovalent adducts do not survive conditions within a mass spectrometer.[16]

Annealed Seq-1 gave a single peak at RT 26.0 min, which was collected and identified by MALDI-TOF-MS (Figure 3A). Preincubation of Seq-1 with **2** for 30 min at room temperature followed by immediate injection onto the HPLC column

DOI: 10.1021/ml100131b | ACS Med. Chem. Lett. 2010, 1, 427–432

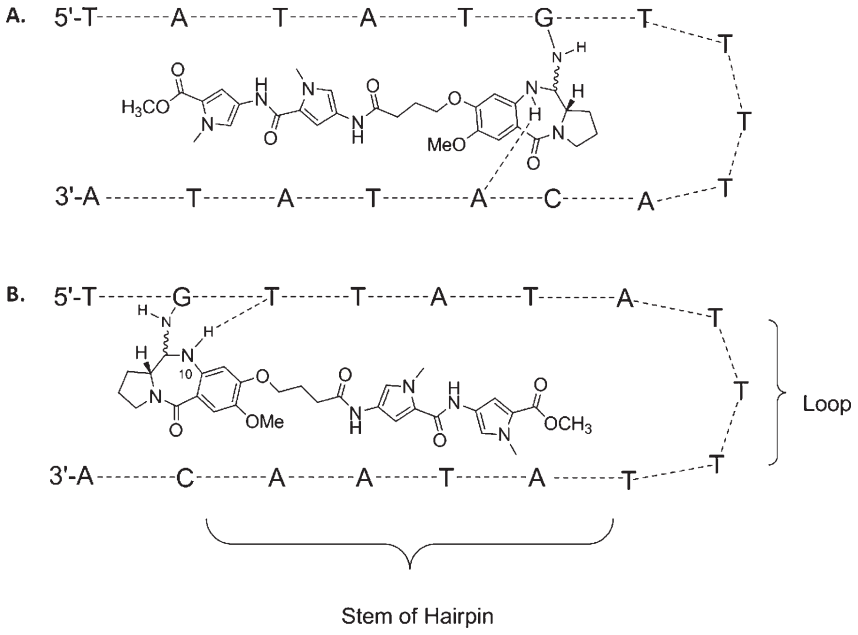

Figure 2. Schematic diagrams showing the most likely orientations of **2** after covalent binding to the two different sets of oligonucleotides. (A) For Seq-1 to Seq-4, **2** is likely to orientate with its A ring in the 5′-direction with a hydrogen bond forming between the N10-H of the PBD and the N3 of an adenine on the opposite strand. (B) For Seq-5 to Seq-8, **2** is more likely to orientate with its A ring in the 3′-direction with a hydrogen bond forming between the N10-H of the PBD and either a thymine (2-carbonyl) or an adenine (N3) base adjacent to the covalently modified guanine.

resulted in the rapid appearance of a new peak at RT 27.0 min (Figure 3B) with near complete disappearance of Seq-1 by 60 min (Figure 3C). The adduct peak (RT 27.0 min) was collected and identified by MALDI-TOF-MS as the 1:1 **2**/Seq-1 covalent adduct (see the Supporting Information). A time−course experiment was carried out by measuring the decreasing area under the curve (AUC) for the Seq-1 peak at various time points (up to 60 min) using the Chromquest program (Thermo Scientific). These data were plotted using the Sigmaplot program (Systat Software), and a rate constant calculated in μM/min based on the reduction in integrated AUC of the oligonucleotide peak as reaction with **2** progressed (see the Supporting Information for details of rate calculations). Similar time−course studies were carried out for the other oligonucleotides (Seq-2 to Seq-8), and the time−course plots and rate constants are shown in Figure 4 and Table 1, respectively. Contrary to literature reports indicating that PBDs have a rank order of preference for 5′-Pu-G-Pu-3′ > Pu-G-Py ~ Py-G-Pu > Py-G-Py sequences,[8,17] the rate of adduct formation for reaction of **2** with Seq-1 to Seq-8 was found to be Py-G-Py > Py-G-Pu > Pu-G-Py/Pu-G-Pu (where Pu = A and Py = T), the opposite to that expected. Furthermore, the preference for TGT was consistent for both binding site locations within the hairpin, suggesting that it is independent of adduct orientation.

To gain insight into these surprising results, molecular models were built of all eight adducts, and free energies were calculated for the noncovalent interaction of **2** with each oligonucleotide using the AMBER (v9) software.[18] According to the model, the higher rate constants obtained for TGT and TGA sequences with the PBD A ring orientated in the 3′-direction (Figure 2B) imply that the N10-proton of the B ring can hydrogen bond to either the N3 of an adenine base on the 3′-side of the covalently modified guanine (Figure 5A) or a similarly placed C2-carbonyl of a thymine base (Figure 5B). However, there appears to be a preference for thymine over adenine (i.e., rates = 0.808 ± 0.008 and 0.196 ± 0.004 μM/min for TGT [Seq-6] vs TGA [Seq-8], respectively). On the other hand, according to the model, with the PBD A ring orientated in the 5′-direction (Figure 2A), the B ring N10-proton can only hydrogen bond with the opposite strand base complementary to the base on the 5′-side of the covalently modified guanine. For the two fastest reacting sequences, TGT (Seq-2) and TGA (Seq-4) (i.e., rates = 0.506 ± 0.012 and 0.364 ± 0.012 μM/min, respectively), this is an adenine in either case. This is the first report of a 5′-oriented PBD monomer adduct and the potential hydrogen bond from the PBD N10-proton to the noncovalently bound strand. However, a similar model has been proposed for the interaction of one PBD unit of the PBD dimer SJG-136 with DNA when forming an intrastrand cross-link.[15]

Finally, the results of the free energy calculations (Table 1), which relate to the thermodynamic stability of the PBD-DNA adducts, are in broad agreement with literature reports that the preferred orientation of a PBD adduct is with the A ring pointing toward the 3′-end of the covalently modified DNA strand. For example, between pairs of oligonucleotides with AGA (Seq-1 and Seq-5), TGT (Seq-2 and Seq-6), AGT (Seq-3 and Seq-7), and TGA (Seq-4 and Seq-8) sequences, lower energies were obtained for the 3′-orientation (i.e., for Seq-5, Seq-6, Seq-7, and Seq-8) in each case. However, these energy differences are small, and the HPLC results clearly demonstrate that PBDs can form 5′-adducts with high reaction rates (e.g., Seq-2 and Seq-4).

DOI: 10.1021/ml100131b | ACS Med. Chem. Lett. 2010, 1, 427–432

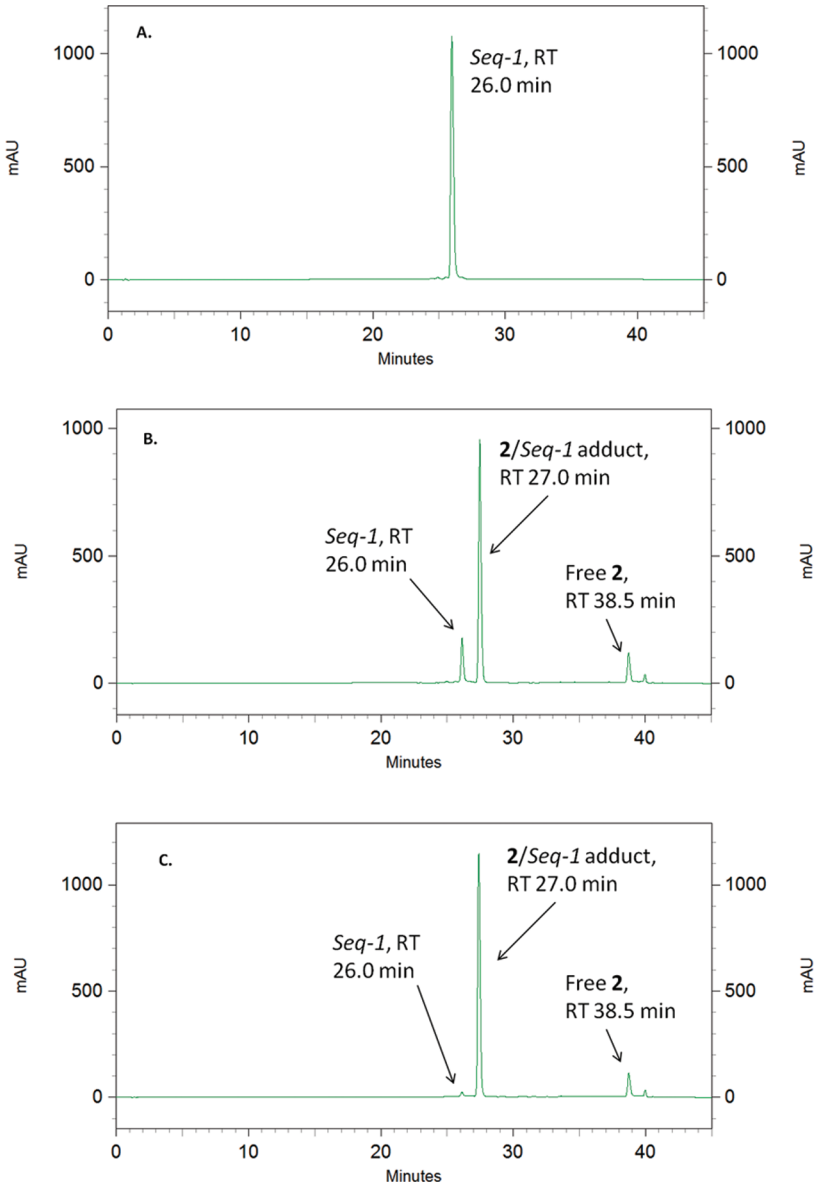

Figure 3. (A) Seq-1 alone at RT 26.0 min. (B) 30 min after mixing Seq-1 with **2**; 95% decrease in Seq-1 peak area at RT 26 min, and appearance of the Seq-1/**2** adduct at RT 27.0 min. (C) As in panel B but 60 min after mixing when almost complete reaction had occurred.

In conclusion, this is the first report of comparative kinetic data for the reaction of a PBD monomer with isomeric oligonucleotides of varying sequence. Such data cannot be obtained from gel-based methods and is only made possible through the rapid sampling achievable with HPLC and the use of MS for confirmation of adduct structures. The results are consistent with the literature in that, based on the kinetic data, **2** appears to have a kinetic preference for binding to DNA with its A ring oriented toward the 3′-end of the covalently modified strand. However, within 60 min, all eight oligonucleotides had fully reacted, thus demonstrating that both 3′- and 5′-adducts may be important from a biological standpoint. Furthermore, contrary to the literature, the results demonstrate that, at least for the sequences studied, a thymine rather than an adenine base is preferred on the A ring side of the covalently modified guanine. This may be explained by our model, which shows that the C2-carbonyl oxygen atom of thymine resides in the same position in space as the N3 of adenine. Taken together, these findings are significant in providing further understanding at the molecular level of how PBD molecules interact with DNA, and should assist in the interpretation of results from gel-based assays such as DNA footprinting, in vitro transcription stop, and EMSA.

EXPERIMENTAL PROCEDURES

Interaction of **2** with oligonucleotides Seq-1 to Seq-8 was studied with an HPLC assay utilizing a X-Terra MS C18 2.5 μM column (4.6 mm × 50 mm) and a gradient of 40% acetonitrile/water and 100 mM TEAB/water as mobile phase with a flow rate of 0.5 mL/min and UV detection at 254 nm.

DOI: 10.1021/ml100131b | *ACS Med. Chem. Lett.* **2010**, *1*, 427–432

A 4:1 molar ratio of **2**:oligonucleotide was used, with each single-stranded oligonucleotide dissolved in 1 M ammonium acetate to form stock solutions of 1 mM. The hairpin oligonucleotides were initially annealed by heating their 1 mM solutions to 70 °C for 10 min followed by gradual cooling over 8 h and storage overnight at −20 °C. Working solutions of oligonucleotides of 50 μM were then prepared by diluting the annealed stock solutions with 100 mM ammonium acetate. GWL-78 (**2**) was obtained from Spirogen Ltd (Batch No. SG2274.005) and was dissolved in methanol to form a stock solution of 10 mM, which was stored at −20 °C for no longer than 4 months. Working solutions of **2** of 200 μM were prepared by diluting the above solution with 100 mM ammonium acetate. The working solution of **2** was added to the working solution of an oligonucleotide at room temperature, and the mixture was incubated for different time intervals at room temperature. After injection of reaction mixtures and controls onto the HPLC column at different time intervals, fractions containing peaks were collected, combined where appropriate, lyophilized, and subjected to MALDI-TOF analysis to identify constituents (see the Supporting Information for detailed methodology).

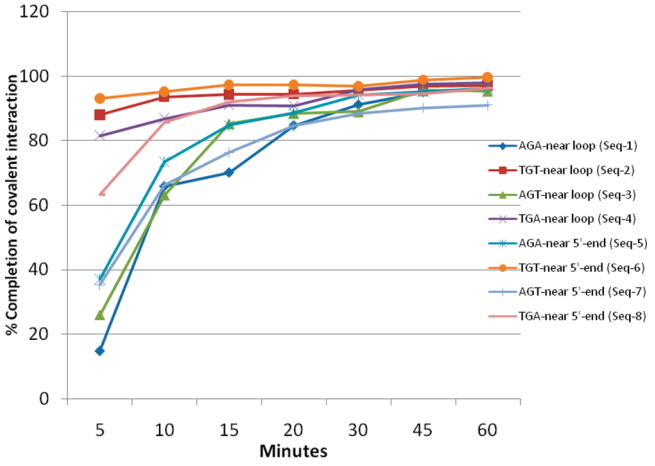

Figure 4. Time course of reaction of **2** with the eight oligonucleotides Seq-1 to Seq-8 (Table 1) measured by reduction of AUC for each oligonucleotide with time. Although all oligonucleotides reacted completely within 60 min, those containing a 5′-TGT binding site at either location (i.e., Seq-2 and Seq-6) reacted the fastest followed closely by Seq-4 (5′-TGA), which was slightly less reactive than Seq-2.

An Applied Biosystems Voyager DE-Pro Biospectrometry Workstation MALDI-TOF mass spectrometer (Framingham, MA) was used to obtain MALDI-TOF spectra of components within lyophilized HPLC fractions. Samples from fractions containing single components were prepared by diluting with matrix (37 mg THAP in 1 mL of acetonitrile, 45 mg of ammonium citrate in 1 mL of water, mixed 1:1 for matrix) either 2:1, 1:1, or 1:5 (sample:matrix) prior to MALDI analysis. One microliter of sample was spotted onto the MALDI target plate and allowed to dry. Samples were analyzed in positive linear mode using delayed extraction (500 ns) and an accelerating voltage of 25000 V. Acquisition was between 4000 and 15000 Da with 100 shots/spectrum.

Molecular models were constructed using the AMBER(v9) modeling software, and dynamics simulations were performed using the AMBER "Sander" program. For each oligonucleotide, dynamics simulations were carried out over 2 ns at room temperature (300 K), and free energy was calculated using the AMBER MM_PBSA approach.[18] In this method, internal energies and nonbonded interactions (long-range cutoff) derived from molecular mechanics were combined with the generalized Born continuum solvent method. Structures used for the free energy calculations for each oligonucleotide were based on 190 models assessed at equal intervals during the molecular dynamics simulations. At certain phases during the simulations, the terminal A-T base pairs of all oligonucleotides showed a temporary loss of hydrogen bonding and became separated to some extent. This occurred at the terminal positions because of the lack of additional stabilizing forces from neighboring bases. Flexing motions within the DNA strands also contributed to this effect. However, no additional restraints were imposed during the dynamics simulations to ameliorate these instabilities.

SUPPORTING INFORMATION AVAILABLE

Experimental procedures and relevant data for HPLC, MS and modelling. This material is available free of charge via the Internet at http://pubs.acs.org.

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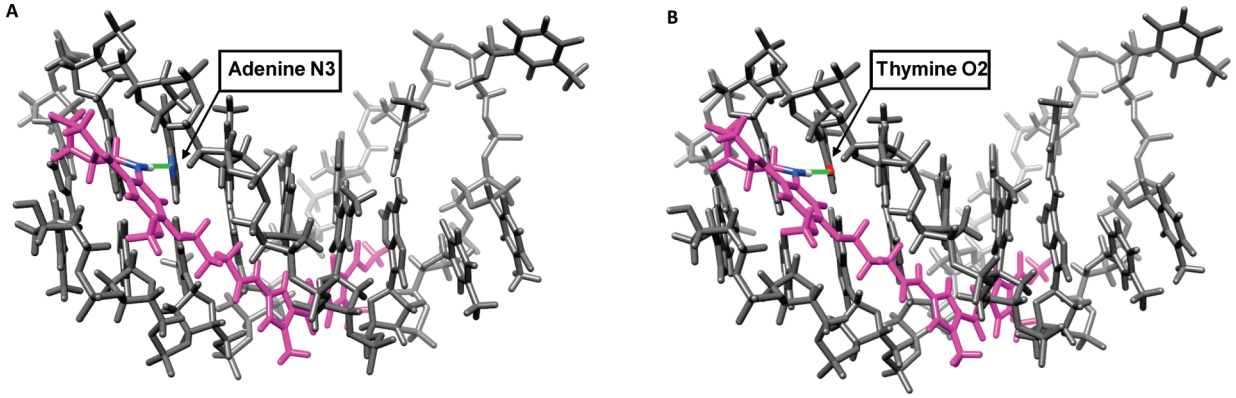

Figure 5. Energy minimized molecular models of **2** covalently attached to Seq-5 (A) and Seq-6 (B) according to the arrangement indicated in Figure 2B, showing the possible hydrogen-bonding interactions of the PBD N10-H proton with the two different bases on the 3′-side of the covalently modified guanine. (A) With Seq-5, hydrogen bonding may occur with the N3 of adenine. (B) With Seq-6, hydrogen bonding may occur with the oxygen atom of the C2-carbonyl group of thymine. DNA is depicted in gray, **2** is in magenta, and the hydrogen bonds are shown in green.

DOI: 10.1021/ml100131b | *ACS Med. Chem. Lett.* **2010**, *1*, 427–432

Funding Sources: We thank the Commonwealth Commission for a Scholarship for KMR (BDCA005/01). Spirogen Ltd. is acknowledged for providing the GWL-78 (**2**) and funding for the oligonucleotides.

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DOI: 10.1021/ml100131b | *ACS Med. Chem. Lett.* **2010,** *1*, 427–432