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A bulge binding agent with novel wedge-shape topology for stimulation of DNA triplet repeat strand slippage synthesis

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ABSTRACT

Expansion of DNA repeat sequences is associated with many human genetic diseases. Bulged DNA structures have been implicated as intermediates in DNA slippage within the DNA repeat regions. Herein a bulge binding agent with novel wedge-shape topology of the aromatic moiety was designed and synthesized. The compound–bulge DNA interactions were characterized via UV melting experiments, circular dichroism and were quantitated by surface plasmon resonance with K_d of 41.5 μM. This compound showed remarkable stimulation for DNA triplet repeat strand slippage synthesis in vitro.

Bulge structure in nucleic acid has been shown to play a significant biological role in protein binding recognition,[1] frameshift mutation,[2] imperfect homologous recombination by repair enzymes,[3] naturally occurring antisense RNA,[4] and expansion of triplet repeats during DNA synthesis.[5] Goldberg group have reported a series of bulge binding molecules designed from the decomposed product of NCS-chrom.[6] NCS-chrom is a bicyclic enediyne antibiotic.[7] One of its decomposed products NCSi-gb shows bulge binding selectivity and its solution structure with bulge DNA has been reported.[8] Some other similar spirocyclic molecules such as DDI (double-decker intercalator) and ent-DDI have been designed and synthesized, which are more stable compared with NCSi-gb and show DNA slippage stimulation effect in vitro.[6] The capacity for DDI to recognize bulges stems specifically from the orientation of the ring systems, transposed approximately 60° by the spirolactone, and the right-handed 35° twist of the molecules.[6a] On the other hand, we recently reported binaphthol aminosugars as bulge probe, which could stimulate DNA slippage synthesis in vitro.[9] The helical twist between two naphthyl rings in binaphthol aminosugars is 90°. However, doxorubicin with nearly flat aromatic surface was reported to inhibit the expansion. The topological structure of the aromatic moiety seems to be important for its slippage property. Whether an aromatic-containing aminosugar with relative large dihedron angle θ ($90° < \theta < 180°$) could also act as bulge probe? In order to further study the topological affect of spirocyclic moiety on bulge DNA binding, herein we designed and synthesized a phenothiazine aminosugar **1** with large dihedron angle (~140°) between two benzene rings (Scheme 1). Aminosugar moiety in **1** was employed to enhance its aqueous solubility and binding to the phosphate backbone at the bulged site. This phenothiazine aminosugar was found to act as DNA bulge binders and stimulate DNA slippage synthesis in vitro.

As shown in Scheme 2, compound **1** was prepared from phenothiazine based on simple two-step organic synthesis. Phenothiazine **2** was firstly converted to **3** using NaH condition.[10] The intermediate **3** was treated with trichloroacetimidate **4**.[11] After gel purification, all protecting groups were removed by sodium methoxide in methanol to give **1**. Compound **1** was structurally characterized by 1- and 2-D NMR spectroscopy, including COSY, HSQC, HMBC, and ROESY experiments, in conjunction with mass spectrometry (see SI for NMR spectra).

Circular dichroism (CD) spectrum was used to test the bulge binding selectivity of compound **1**. The DNA sequences used in this study contain B-formed double helix, hairpin-, and bugle-containing DNA structures. Compound **1** has nearly no CD signal in the wavelength scope tested. The DNA concentration used in the experiment is 20 μM and [compound **1**]/[DNA] ratio is 10 for each sequence. Figure 1 shows the effect of compound **1** on the different DNA sequences. When compound **1** was added, the change CD signal of bulge-containing DNA is distinctive, but little change was observed for duplex and hairpin DNA. From the CD spectra, it could be concluded that compound **1** can bind to DNA bulge structure selectively. This result was consistent with the NMR research reported on the selective interactions between phenothiazine derivates and bulge-contained RNA.[12]

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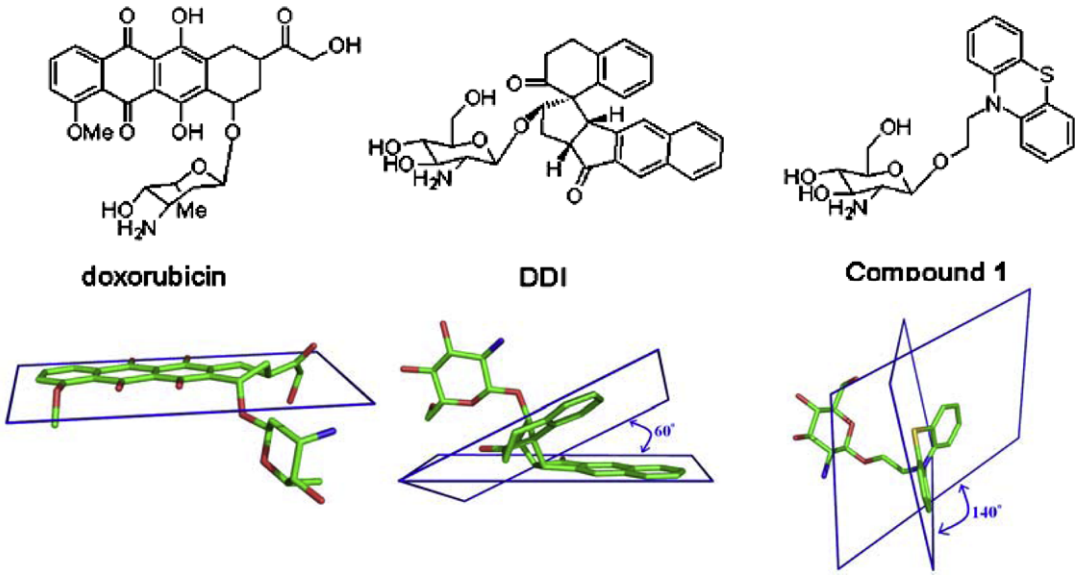
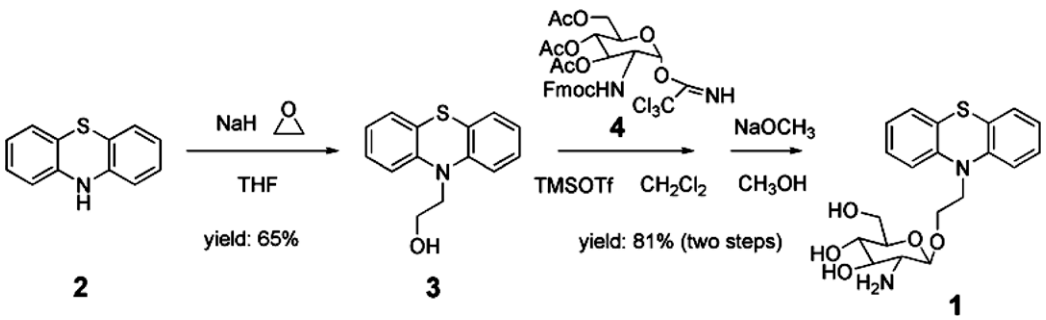


Scheme 1. Aminosugar compounds mentioned. The topological structures of three molecules are different from each other: the aromatic surface of doxorubicin is nearly planar and is more flexible than the other two. The aromatic surfaces of DDI and **1** are fixed and wedge-shaped but they have different angle. The 3D structures were generated using the InsightII molecular modeling program.[16] The structures' energy minimization was performed using the CVFF force field[17] with a 0.001 kcal/mol energy gradient convergence criterion by InsightII/Discover package. Graphics were generated using PyMOL (http://pymol.sourceforge.net).



Scheme 2. Synthesis of compound **1** from phenothiazine.

In order to test the ability of **1** to stabilize the possible bulge intermediate, we measured the melting temperature change of ATT and AAT bugle-contained DNA sequences by adding compound **1** (Fig. S1). The melting temperature of ATT bugle-containing DNA changed from 35.8 to 36.6 °C by adding the compound and that for AAT bugle-containing DNA from 35.7 to 37.5 °C, which implying that compound **1** can stabilize both AAT and ATT bulge structure.

Surface plasmon resonance (SPR) measurements were further made to quantitate DNA–drug interactions. As shown in Figure 2, the steady state response for compound **1** binding to bulged DNA surface was plotted versus the analyte concentration. The data were fitted to a simple Langmuir isotherm for bimolecular interactions: $R_{eq} = R^{*}_{max}([A]/(K_d + [A]))$, wherein R_{eq} is the measured response, [A] is the concentration of drug, R_{max} is the maximum response at saturation of surface binding sites, and K_d is the dissociation constant.[13] Compound **1** showed moderate binding affinity (41.5 ± 5.0 μM) with ATT bulge-containing structure, which was comparable with K_d values reported for wedge-shape bulge binding agents.[6c] However, the binding response for hairpin (K_d = 140 ± 40 μM) and double strand (K_d = 170 ± 10 μM) structures are significantly smaller than that for ATT bulge-containing structure. Herein SPR-based assay works simple, efficient, and rapid for bulged DNA–drug interactions, which should facilitate the study on DNA slippage synthesis mechanism and as a method for high-throughput screening of bulge binders.

As bulge has been postulated as intermediate in DNA slippage synthesis involving templates with nucleotide repeats, we examined the influence of compound **1** on the triplet repeat expansion in vitro. The AAT·ATT trinucleotide repeats distribute in different types of genomic sequences,[14] which is one of the most instable triplet repeat sequences reported to slip in vitro.[15] DNA slippage synthesis was measured at 23 °C using the primer/template oligomers of $(ATT)_3$ and $(AAT)_5$ in a reaction catalyzed by the Klenow fragment of DNA polymerase I lacking 3′–5′ exonuclease activity. The primer was 5′-^{32}P-end-labeled using [γ-^{32}P] ATP and T4 polynucleotide kinase. Dose-dependency experiment was carried out by changing the concentration of **1** from 4 to 400 μM. The experimental result was analyzed by electrophoresis (Fig. 3). In the control reaction (Fig. 3, lane 2), the primer was expanded to an average band length of 17 bp (base pair), a little longer than 15 bp expected, which indicated that the slippage occurred during DNA synthesis. The slippage could be restrained by the addition of doxorubicin (Fig. 3, lane 3).[6c] When compound **1** was hold at a relative

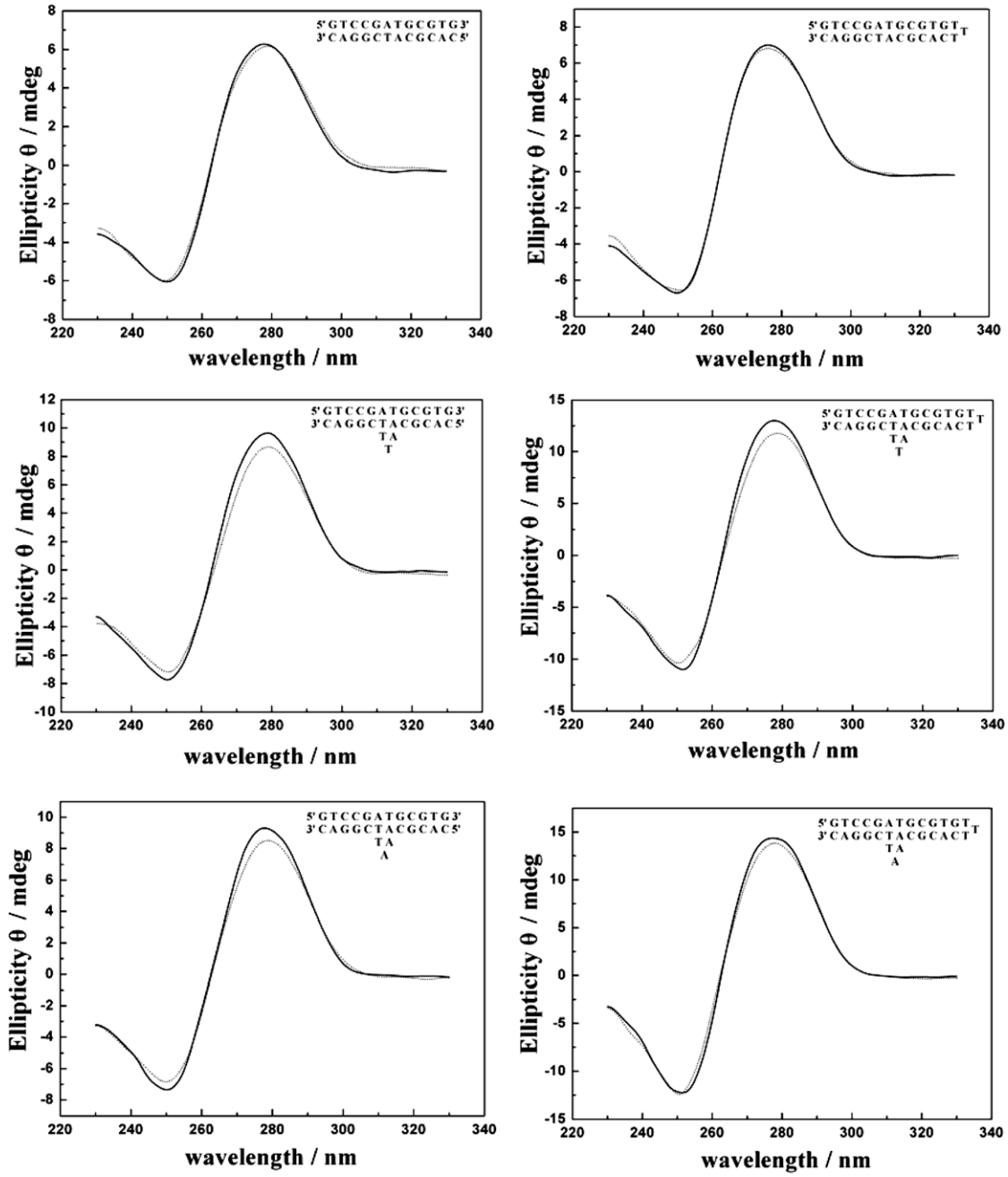


Figure 1. Circular dichroism spectra of DNA (20 μM) alone (solid line) and **1**–DNA complex after addition of **1** (200 μM) (dashed line). The CD spectra were performed on a Jasco-715 spectropolarimeter, using quartz cylindrical cell of 1 mm path length. The cell compartment was continuously purged with dry N_2. Data were recorded at a bandwidth of 1.0 nm and measured at every 0.2 nm over of 230–330 nm at 20 °C in phosphate buffer containing 50 μM NaCl,10 μM phosphate, pH = 7.0. All oligonucleotides (20 μM) were heated to 95 °C for 1 min and then cooled to the 15 °C slowly before use.

low concentration, little affect was detected (Fig. 3, lanes 4 and 5). The stimulating effect of **1** became distinctive at higher concentration. The average band length of slippage[15] increased with the concentrations of **1**, such as 18, 20, 32, 71 bp for 50, 100, 200, 400 μM, respectively. The enhancement of DNA slippage synthesis by compound **1** could also be restrained by the addition of doxorubicin (Fig. 3, lane 10). Phenothiazine or aminosugar alone did not show any detectable DNA bulge selective binding, nor stimulate DNA slippage (data not shown). The experimental result demonstrated that compound **1** having different aromatic topology against DDI could also stimulate the DNA slippage synthesis in vitro. Comparing the electrophoresis result with the binding affinity revealed by SPR, we propose that the trinucleotide repeats DNA slippage synthesis stimulated by small molecule could be a dynamic equilibrium, so molecule with moderate bulge-binding affinity can give well slippage result.[9] Compared with the stimulation effect of DNA strand slippage for DDI[6c] or binaphthol aminosugars,[9] the title compound **1** exhibits relative smaller effect at low concentration (<100 μM). However, with enhancement of the concentration to 400 μM, compound **1** has similar effect with that of DDI, where the triplet expansion nearly reaches saturation. Further work is also needed to clearly elucidate the stimulation ability of DNA stand slippage for compound **1**.

In summary, we have synthesized and characterized a phenothiazine aminosugar compound with novel wedge-shaped aro-

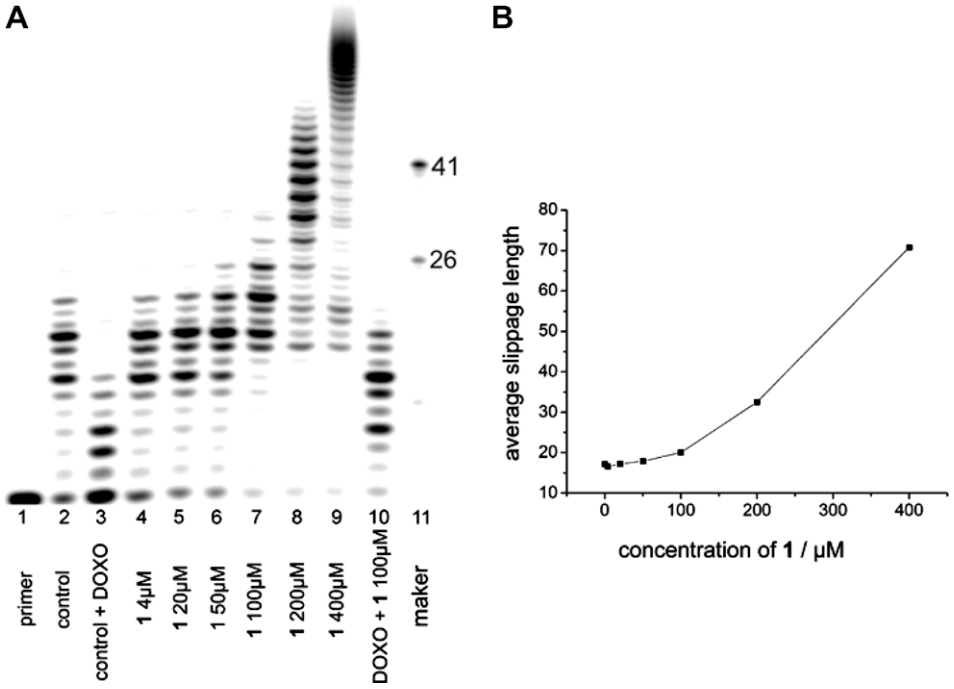


Figure 3. (A) Dose effects of compound **1** on the expansion of the ATT·AAT trinucleotide repeat. A standard reaction (23 °C, 12 h) containing 5′-^{32}P-end-labeled $(ATT)_3$ and unlabeled template $(AAT)_5$ was catalyzed by the Klenow fragment of *Escherichia coli.* DNA polymerase. The products were analyzed on 15% denaturing PAGE gel. Lane 11 is marker, 42 bp (top) and 26 bp (bottom); lane 1 is the reaction without Klenow fragment; lane 2 is the reaction with 2% DMSO as control; lane 3 is the control reaction mixed with doxorubicin 40 μM; lanes 4–9 is reaction in the presence of **1** from 4, 20, 50, 100, 200 to 400 μM, respectively, and lane 10 is the reaction containing both compound **1** (100 μM) and doxorubicin (40 μM). (B) The average slippage length is calculated as the total (slippage length * its intensity)/the total band intensity of each lane.

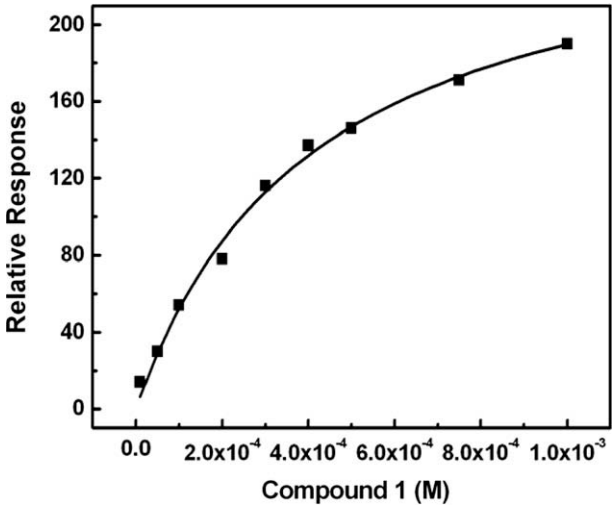


Figure 2. Steady state binding responses versus concentrations for compound **1** binding to bulged DNA surface. Concentrations of **1** ranged from 10 μM to 1 mM. The DNA surface immobilization level was about 1800 RUs. Solid line represents the best fitting result with K_d = 41.5 ± 5.0 μM, R_{max} = 268.4 ± 13.3 RUs, R^2 = 0.9939. See detailed experiments in Supporting information.

matic rings. This compound was easily prepared from available reagents. It exhibited bulge binding selectivity and could stimulate DNA slippage synthesis. The bulge DNA and drug interactions were quantitated by SPR-based method. This methodology may prove to be useful in drug development and serve as a high-throughput assay for screening of bulge binders. The title compound also provides more molecular choices to control DNA triplet repeat expansion in vitro.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.10.038.

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