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N,*N*′-Bis(3-aminopropyl)-2,7-diamino-1,8-naphthyridine stabilized a single pyrimidine bulge in duplex DNA

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Abstract—We here show the first identified ligand 2,7-diamino-1,8-naphthyridine (DANP) that strongly and specifically binds to the single cytosine and thymine bulges with exclusively 1:1 stoichiometry.

Small molecules that bind to bulges and mismatches in duplex DNA and those covalently attached to DNA are important probes for the dynamics of unusual DNA structures.[1,2] With a combination of bulge- and mismatch-forming hybridization, these molecules are also useful for the detection of base mutation and deletion.[3,4] Toward this end, we have investigated a series of compounds that have hydrogen-bonding surface fully complementary to that of the unpaired or partially paired nucleotide bases, and found that 2-amino-1,8-naphthyridine and 8-azaquinolone effectively functioned as a molecular element for the recognition of guanine and adenine bases, respectively.[5,6] For the cytosine, Teramae and co-workers used 2-amino-1,8-naphthyridine and proposed formation of two hydrogen bonds to the cytosine.[7] We have independently reported that a dimeric form of 2-amino-1,8-naphthyridine strongly stabilized C–C mismatches.[8] Because these ligands bound to the bulge and mismatched structure in duplex DNA, the equilibrium between single strands and a duplex shifted toward the duplex state, making the apparent stability of the duplex increase. In the study, we proposed a formation of three hydrogen bonds between a protonated form of 2-amino-1,8-naphthyridine and the cytosine. As protonation of the nitrogen in the heterocycles effectively modulated the hydrogen-bonding surface of the molecule, further studies were carried out to see the scope and limitation of the idea. We here describe a new molecular probe *N*,*N*′-bis(3-aminopropyl)-2,7-diamino-1,8-naphthyridine (DANP) that could stabilize not only a single cytosine but also the thymine bulge in duplex DNA (Chart 1). Cold spray ionization time-of-flight mass spectrometry showed that DANP bound to the cytosine and thymine bulges with a 1:1 stoichiometry. The pH dependency of UV spectra of 2,7-diamino-1,8-naphthyridine supported the hydrogen bonding of DANP to the cytosine and thymine through the protonated form.

We have focused on the 2,7-diamino-1,8-naphthyridine, because it has an alignment of hydrogen-bonding groups in the order of a donor (D), acceptor (A), acceptor, and donor. We anticipated that rich hydrogen-bonding groups in one edge of a planar aromatic ring provide a good chance to form stable complex with bulged nucleotides in duplex DNA. DANP was obtained by nucleophilic substitution of 2,7-dichloro-1,8-naphthyridine[9] with 1,3-propandiamine (Scheme 1). Since the initially formed DANP was difficult to isolate from 1,3-propandiamine used as a solvent, the crude

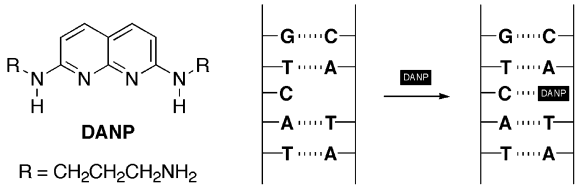

Chart 1. Structure of DANP and an illustration of DANP mediated stabilization of C-bulge.

Keywords: Cytosine; Bulge; CSI-TOF; DNA.
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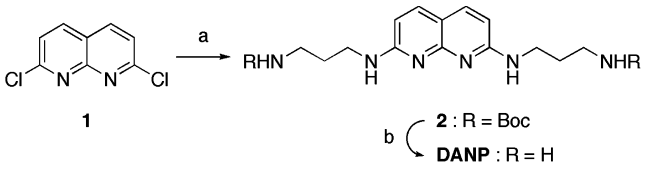

Scheme 1. Reagents and conditions: (a) 1,3-diaminopropane, 60 °C, 24 h, then $(Boc)_2O$, $CHCl_3$, 53%; (b) HCl, EtOAc, $CHCl_3$, quantitative.

product was treated with di-*tert*-butyl dicarbonate to produce Boc-protected DANP. After purification by silica gel column chromatography, the Boc protecting group was removed to give DANP.

The binding of DANP to DNA containing a single nucleotide bulge was examined by measuring melting temperature (T_m) of duplexes 5′-d(TCCA**X**_**Y**CAAC)-3′/3′-d(AGGT**ZNW**GTTG)-5′ containing a single nucleotide bulge **N**, where **N** was adenine, guanine, cytosine, or thymine. The **X**–**Z** and **Y**–**W** were any combinations of Watson–Crick base pairs. Duplexes containing the cytosine bulge in all 16 flanking sequences [4(**X**–**Z**) × 4(**Y**–**W**)] were significantly stabilized as judged by the increase of T_m (ΔT_m) in the presence of DANP (100 μM) (ΔT_m = 7.0–12.4 °C) (Fig. 1). The largest ΔT_m (12.4 ° C) was recorded for the duplex 5′-d(TCCA-T_ACAAC)-3′/3′-d(AGGTACTGTTG)-5′ (**T_A/ACT**) where the cytosine bulge was flanked by T–A and A–T base pairs. It was also shown that thymine bulges were effectively stabilized by DANP binding (ΔT_m = 4.7–9.4 °C). In contrast, the T_m increases of guanine (ΔT_m = 3.2–5.6 °C) and adenine (ΔT_m = 2.3–5.7 °C) bulges were less significant as compared to that observed for the cytosine and thymine bulges. It is noteworthy that fully matched duplexes 5′-d(TCCA**XY**CAAC)-3′/3′-d(AGGT**ZW**GTTG)-5′, where **X**–**Z** and **Y**–**W** were any combinations of Watson–Crick base pairs were only weakly stabilized (ΔT_m = 0.5–2.0 °C) under the set conditions (data not shown). These results indicated that DANP showed a preference for the binding to the single pyrimidine bulge over the purine bulge and fully matched duplexes.

The stoichiometry for the binding of DANP to the cytosine and thymine bulge was examined by cold spray ionization time-of-flight mass spectrometry (CSI-TOF MS).[10] In the presence of two molar equivalents of DANP to the cytosine bulge duplex **T_A/ACT**, a distinct ion at *m*/*z* of 1323.81 and 1654.96 corresponding to 5^- and 4^- ion, respectively, of a 1:1 complex was detected in addition to the intact duplex (Fig. 2). Further increase of the DANP concentration to 6 equiv resulted in an increase of the ion intensity of the 1:1 complex with a concomitant decrease of the intact duplex. Under the conditions, complexes with other binding stoichiometries could not be detected. Similarly, CSI-TOF MS showed a 1:1 complex between DANP and the thymine bulge duplex 5′-d(TCCAT_ACAAC)-3′/3′-d(AGG-TATTGTTG)-5′ (**T_A/ATT**) (Fig. 3). With increasing the amount of DANP, the ion corresponding to the 1:1 complex (*m*/*z* 1326.42) increased with a concomitant decrease of the ion corresponding to the intact duplex

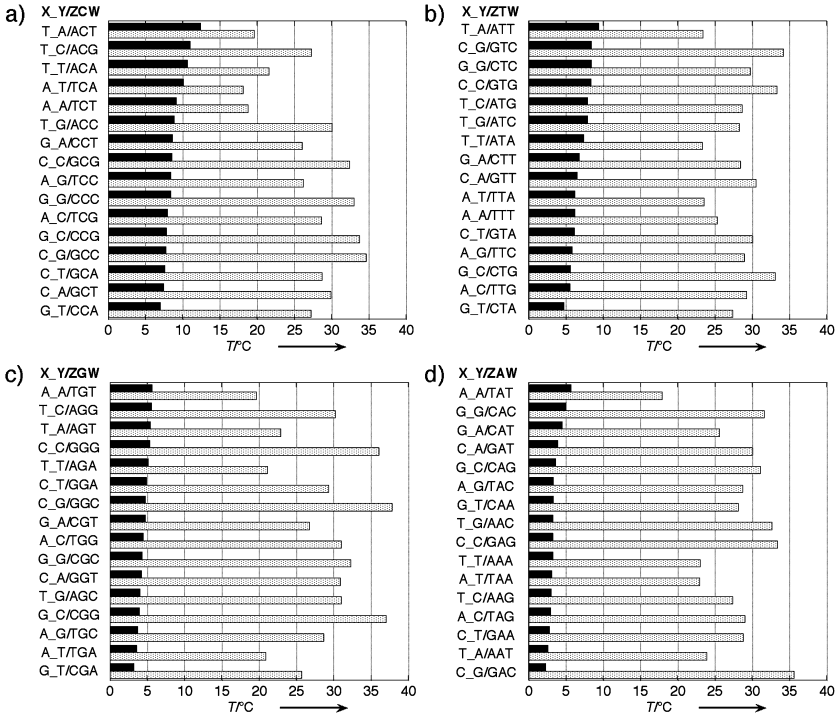

Figure 1. Effects of DANP–binding on the melting temperatures of 5′-d(TCCA**X**_**Y**CAAC)-3′/3′-d(AGGT**ZNW**GTTG)-5′ containing a single nucleotide bulge (**N**) flanked by **X**–**Z** and **Y**–**W** base pairs. Melting temperature (T_m) of duplex (4.8 μM) measured in sodium cacodylate buffer (pH 7.0, 10 mM) and sodium chloride (100 mM) was shown with a gray bar. Increase of T_m (ΔT_m) in the presence of DANP (100 μM) was shown with a black bar. The flanking base pairs (**X**–**Z** and **Y**–**W**) were shown in the left of the bar. Key: (a) C bulge (**N** = C), (b) T bulge (**N** = T), (c) G bulge (**N** = G), and (d) A bulge (**N** = A).

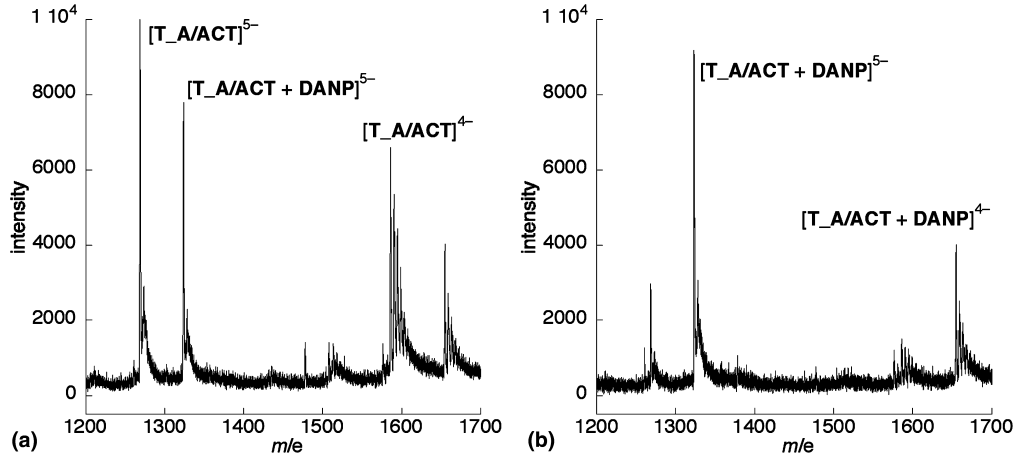

Figure 2. CSI-TOF MS of 5′-d(TCCA**T**_**A**CAAC)-3′/3′-d(AGGT**ACT**GTTG)-5′ (**T_A/ACT**) (20 μM) measured in 50% aqueous methanol and 100 mM ammonium acetate in the presence of (a) 40 and (b) 120 μM of DANP.

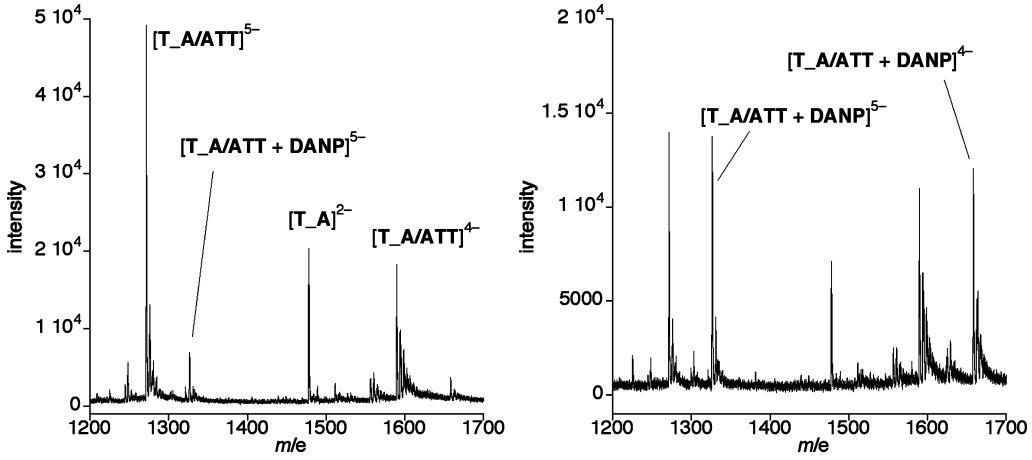

Figure 3. CSI-TOF MS of 5′-d(TCCA**T**_**A**CAAC)-3′/3′-d(AGGT**ATT**GTTG)-5′ (**T_A/ATT**) (20 μM) measured in 50% aqueous methanol and 100 mM ammonium acetate in the presence of (a) 40 and (b) 120 μM of DANP.

(*m*/*z* 1271.57). These CSI-TOF MS experiments strongly suggested that DANP bound to both cytosine and thymine bulge with a 1:1 stoichiometry. In the presence of 6 equiv of DANP, the ion intensity corresponding to the intact duplex was much higher for the thymine bulge than for the cytosine bulge, suggesting that DANP binding to the cytosine bulge would be stronger than to the thymine bulge. This is consistent with a larger ΔT_m observed for the cytosine bulge than for the thymine bulge in the same **T_A/ANT** (**N** = C or T) sequence context. The association constant for the DANP binding to the cytosine bulge was determined to be $7.5 \times 10^5\ M^{-1}$ by fluorescence titration and Scatchard plot analysis (data not shown).

To gain further insight into the DANP binding to the cytosine and thymine bulges, the pH dependency of DANP absorption spectra were examined. Absorption spectra of DANP free in solution were sensitive to the pH of the solution (Fig. 4a). At pH 8.5, the absorption maximum was observed at 365 nm, whereas it shifted to 376 nm at pH 5.0. A plot of the absorbance at 376 nm against the solution pH showed a sigmoid curve (Fig. 4b). The pH dependency of the UV absorbance of DANP was rationalized by a protonation at the nitrogen in a 2,7-diamino-1,8-naphthyridine chromophore. Energy calculation in an aqueous solvent using the Cramer–Truhlar solvation methods with density functional theory (B3LYP/6-31G(d)) indicated that a protonation at *N*1 of the 2,7-diamino-1,8-naphthyridine chromophore was energetically much more favorable by 6.9 kcal/mol than a protonation at the 2-amino group. A pK_a of 6.8 for the protonated form of DANP ($DANPH^+$) was obtained as the pH at the inflection of the curve. A protonation of *N*1 of DANP resulted in the modulation of the hydrogen-bonding surface from an alignment of D–A–A–D in DANP to a D–A–D–D alignment in $DANPH^+$. The D–A–D–D alignment of the hydrogen-bonding groups in $DANPH^+$ is fully complementary not only to that of cytosine (D–A–A) but also to that of thymine base (A–D–A). The complementarily hydrogen bonding surface to that of the cytosine and thymine is most likely to rationalize the selective binding of DANP to pyrimidine bases. In a proposed hydrogen-bonding scheme between $DANPH^+$ and cytosine, the proton attached *N*1 would be bound by *N*3 of

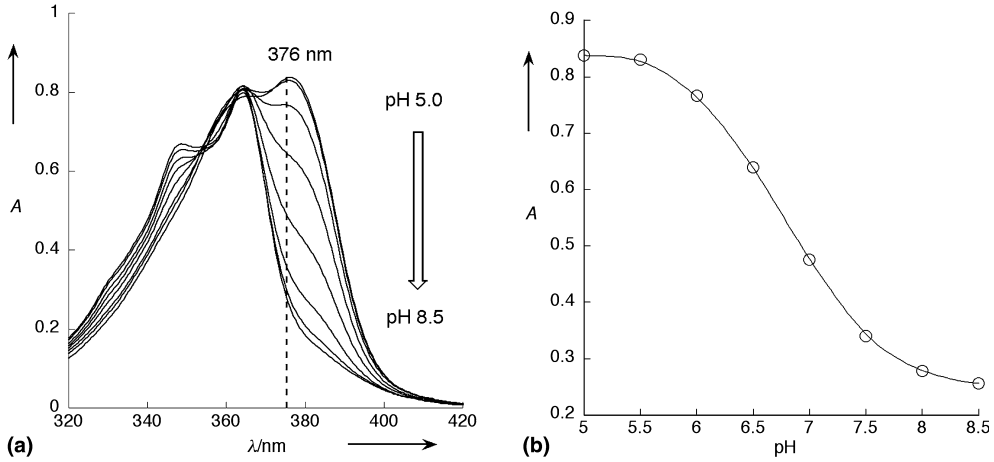

Figure 4. (a) Absorption spectra of DANP (100 μM) recorded at pH of 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 in sodium phosphate buffer (10 mM) and sodium chloride (100 mM). (b) A plot of absorption at 376 nm against the solution pH.

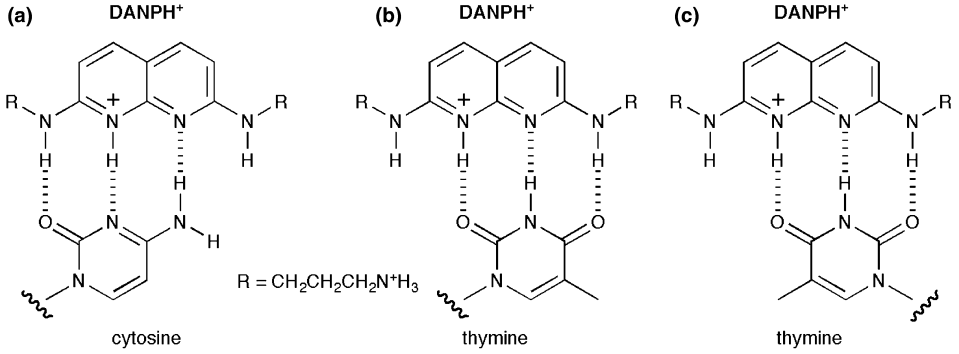

Figure 5. Proposed hydrogen-bonding schemes between $DANPH^+$ and pyrimidine nucleobases. Key: (a) cytosine, (b) and (c) thymine. Two orientations are conceivable for hydrogen bonding of $DANPH^+$ to thymine.

the cytosine. Due to a palindromic A–D–A alignment of hydrogen-bonding groups in the thymine base, two orientations were conceivable for the hydrogen bonding to $DANPH^+$. The difference in the two orientations was that the non-hydrogen bonding amino group was located either in a minor groove (Fig. 5b) or in a major groove of the $DANPH^+$–DNA complex (Fig. 5c).

When aromatic molecules intercalated into DNA, the electronic state of the molecule would be significantly affected by the stacking interaction with the neighboring base pairs. It was confirmed that DANP showed a large spectroscopic change upon binding to a single pyrimidine bulge. The UV absorption was measured with a constant DANP (10 μM) and DNA (30 μM) concentration in phosphate buffer (pH 7.0) and 100 mM NaCl. Free DANP in phosphate buffer (pH 7.0) showed absorption maximum at 364 nm with a shoulder at 376 nm. The presence of fully matched duplex as well as adenine bulge duplex resulted in a small hypochromic shift but not a bathochromic shift of the absorption maximum (Fig. 6a). In contrast, the cytosine bulge duplex (**T_A/ACT**) induced a bathochromic shift by 30 nm to 394 nm with a concomitant hypochromic shift by 65%. Bathochromic shift of DANP absorption was also induced by the thymine bulge (**T_A/ATT**) producing the absorption maximum at 390 nm. Guanine bulge duplex was intermediate between the pyrimidine and adenine bulge duplex in terms of the bathochromic shift. In response to the bathochromic shift, fluorescence spectra of DANP showed a significant change with respect to the emission wavelength and the shape of the spectra. The fluorescence spectra of DANP free in solution excited at its absorption maximum showed an emission maximum at 394 nm in pH 7.0. The emission maximum of DANP was not virtually affected by the presence of fully matched duplex and adenine bulge duplex. However, a broad emission with the emission maximum at 424 nm was observed in the presence of the cytosine bulge. In the presence of thymine bulge, a similar fluorescence spectrum was obtained with a decreased intensity. Quantum yield of DANP fluorescence at 424 nm obtained by an excitation at 394 nm in the presence of **T_A/ACT** and **T_A/ATT** were 0.332 and 0.166, respectively.

The results described here showed that DANP bound not only to the cytosine but also to the thymine bulge. The selective DANP binding to these pyrimidine bulges was most plausible by the protonation of the nitrogen in the 2,7-diamino-1,8-naphthyridine chromophore, producing hydrogen-bonding surfaces fully complementary to those of cytosine and thymine. We have demonstrated that molecules having hydrogen-bonding surface

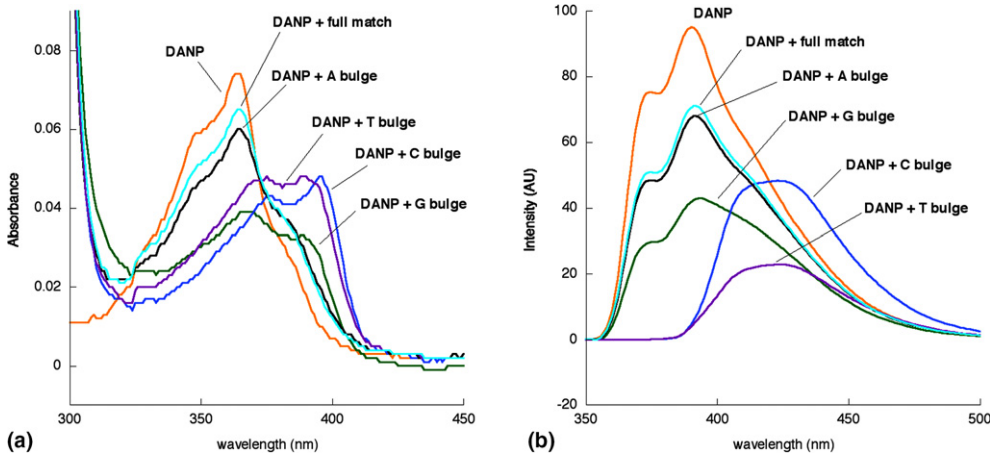

Figure 6. (a) Absorption spectra of DANP (10 μM) recorded in the presence of duplexes 5′-d(TCCAT_ACAAC)-3′/3′-d(AGGTANTGTTG)-5′ containing **N** bulge (**N** = C, T, G, and A) (30 μM) and 10-mer fully matched duplexes 5′-d(TCCATACAAC)-3′/3′-d(AGGTATGTTG)-5′ (30 μM) in sodium phosphate buffer (pH 7.0, 10 mM) and sodium chloride (100 mM). (b) Fluorescence spectra of DANP recorded under the same conditions. Excitation wavelength was at the absorption maxima.

fully complementary to the nucleotide bases were useful probes for the detection of bulges and mismatches in duplex DNA. Because, protonation of the nitrogen in heterocycles could effectively modulate the hydrogen-bonding surface from the acceptor to the donor, the idea of the protonation of the chromophore may provide a new way for the design of a molecular element for the base recognition. Furthermore, the observed spectroscopic changes upon binding of DANP to pyrimidine bulges could be applicable to the detection of the single pyrimidine bulge in duplex DNA.

1. Experimental

1.1. {3-[7-(3-{*tert*}-Butoxycarbonylamino-propylamino)-[1,8]naphthyridin-2-ylamino]-propyl}-carbamic acid *tert*-butyl ester (*2*)

A mixture of **1** (50 mg, 0.25 mmol) and 1,3-diaminopropane (2 ml, 23.9 mmol) was stirred at 60 °C for 24 h. The solvent was evaporated in vacuo. The residue was dissolved in chloroform (5 ml) and was added $(Boc)_2O$ (300 mg, 1.37 mmol). The mixture was stirred at 40 °C for 6 h. Solvents were evaporated in vacuo and the resulting solid was purified by silica gel column chromatography ($CHCl_3$/CH_3OH and hexane/AcOEt) to give **2** (62.9 mg, 53%) as yellow solids: ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 7.54 (d, 2H, J = 8.8 Hz), 6.34 (d, 2H, J = 8.8 Hz), 3.58 (q, 4H, J = 6.0 Hz), 3.22 (q, 4H, J = 6.0 Hz), 1.78 (tt, 4H, J = 6.0 Hz), 1.44 (s, 18H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ = 159.8, 157.2, 156.6, 137.3, 110.8, 106.7, 79.3, 38.5, 37.9, 30.7, 28.5 MS (ESI), *m/e* 497 [M+Na^+], 475 [M+H^+]; HRMS calcd for $C_{24}H_{39}NaN_6O_4$ [M+Na^+] 497.2852, found 497.2834, $C_{24}H_{39}N_6O_4$ [M+H^+] 475.3033, found 475.2996.

1.2. *N*,*N*′-Bis-(3-amino-propyl)-[1,8]naphthyridine-2,7-diamine (DANP)

To a $CHCl_3$ (1 ml) solution of (14.5 mg, 30.6 μmol) was added ethyl acetate containing 4 N HCl (2 ml). The mixture was stirred at room temperature for 30 min. Solvent was evaporated to dryness to give DANP (quantitative) as yellow solids: ^{1}H NMR (CD_3 OD, 400 MHz) δ = 7.79 (d, 2H, J = 8.8 Hz), 6.61 (d, 2H, J = 8.8), 3.63 (t, 4H, J = 6.8 Hz), 3.42 (t, 4H, J = 7.2 Hz), 2.04 (tt, 4H, 7.2 Hz); ^{13}C NMR (D_2O, 100 MHz) δ = 157.3, 148.5, 140.6, 109.0, 108.1, 38.6, 37.4, 26.6. MS (FAB), *m/e* 275 [(M+H)$^+$]; HRMS calcd for $C_{14}H_{23}N_6$ [(M+′H)$^+$] 275.1984, found 275.1987.

1.3. Measurements of melting temperature of bulge-containing duplexes

DANP (100 μM) was dissolved in a sodium cacodylate (10 mM, pH 7.0) containing bulge duplex (4.8 μM) and NaCl (100 mM). The mixture was heated at 50 °C and cooled slowly to make sure that the starting oligomers is in a duplex state. The thermal denaturation profile was recorded on a SHIMADU UV2550 spectrometer equipped with a SHIMADU TMSPC-8 temperature controller. The absorbance of the sample was monitored at 260 nm from 4 to 70 °C with a heating rate of 1 °C/min.

1.4. CSI-TOF measurements

Samples were prepared by mixing DNA (20 μM) and DANP (40 and 120 μM) in 50% methanol in water containing 100 mM NH_4OAc. Mass spectra were obtained with a JEOL JMS-T100 mass spectrometer equipped with cold spray ion source. Spray temperature was set at −10 °C with a sample flow rate of 10 μL/min.

1.5. UV and fluorescent spectra measurements

UV spectra were recorded on a SHIMADU UV2550 spectrometer. Fluorescent spectra were recorded on a SHIMADU RF-5300PC. DNA samples were prepared in 10 mM sodium phosphate buffer at the designate pH in the presence of 100 mM sodium chloride. Excitation wavelength for the fluorescent measurements was the wavelength at the absorption maximum unless otherwise noted.

References and notes

1. Guest, C. R.; Hochstrasser, R. A.; Sowers, L. C.; Millar, D. P. *Biochemistry* **1991**, *30*, 3271.
2. Stivers, J. T. *Nucleic Acids Res.* **1998**, *26*, 3837.
3. Hawkins, M. E.; Balis, F. M. *Nucleic Acids Res.* **2004**, *32*, e62.
4. Nakatani, K. *ChemBioChem.* **2004**, *5*, 1623.
5. Nakatani, K.; Sando, S.; Saito, I. *Nat. Biotechnol.* **2001**, *19*, 51.
6. Hagihara, S.; Kumasawa, H.; Goto, Y.; Hayashi, G.; Kobori, A.; Saito, I.; Nakatani, K. *Nucleic Acids Res.* **2004**, *32*, 278.
7. Yoshimoto, K.; Nishizawa, S.; Minagawa, M.; Teramae, N. *J. Am. Chem. Soc.* **2003**, *125*, 8982.
8. Kobori, A.; Horie, S.; Suda, H.; Saito, I.; Nakatani, K. *J. Am. Chem. Soc.* **2004**, *126*, 557.
9. George, R. N.; Sam, J. G.; Veronica, K. M.; Frank, R. F.; Giacomo, C. *J. Org. Chem.* **1981**, *46*, 833.
10. Yamaguchi, K. *J. Mass. Spectrom.* **2003**, *38*, 473.