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1,8-Naphthyridin-2,7-(*1,8H*)-dione is an Effective Mimic of Protonated Cytosine in Peptide Nucleic Acid Triplex Recognition Systems

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Abstract—A novel bicyclic mimic of protonated cytosine [1,8-naphthyridin-2,7-(*1,8H*)-dione, (K)] for Hoogsteen type triplex recognition of guanine has been designed for incorporation into peptide nucleic acids. Bis-PNA clamps with the K base incorporated in the Hoogsteen strand showed a significant stabilization of the triplexes at pH 7 as compared to similar triplexes with PNA oligomers containing either cytosine (6.7 °C per unit) or pseudoisocytosine (1.5 °C per unit). Cooperative stabilization was observed when the K units were placed in adjacent positions (~3 °C per unit).

Inhibition of gene expression by antigene approaches relies on efficient, specific targeting of double stranded DNA under physiological conditions.[1–3] Triplex targeting by oligonucleotides or by their analogues in the parallel motif is dependent on protonation of cytosine *N*3 for efficiency, as dictated by the $C^{+}\cdot$ G-C Hoogsteen base pair (Fig. 1). Likewise triplex invasion by peptide nucleic acids (PNAs)[4,5] requires cytosine N3 protonation.[6,7] For oligonucleotides, a large number of nucleobases have been synthesised and evaluated for their ability to mimic the function of protonated cytosine in an attempt to eliminate this pH dependence; the most prominent members of which are 5-methylcytosine,[8–11] pseudoisocytosine,[12] *N6*-methyl-8-oxoadenine,[13–15] and 8-oxoadenine.[16] While 5-methylcytosine represents a class of cytosine mimics where the basicity of the *N*3 is increased as a result of methyl substitution on C-5, pseudoisocytosine and 8-oxoadenine are 'permanently protonated' analogues of cytosine and therefore able to form triplex structures, virtually independent of pH. For the targeting of dsDNA by helix invading PNAs, the problem of the pH dependence has been addressed by the use of pseudoisocytosine in the Hoogsteen strand to obtain triplex formation almost independent on pH. We have recently reported that substituted 1,8-naphthyridin-2[*1H*]ones are thymine mimics that through increased interbase stacking overlap confer increased duplex stability.[17] Building on these results we now present the synthesis and evaluation of a Boc-PNA monomer containing a novel nucleobase, 1,8-naphthyridin-2,7-(*1,8H*)-dione (K), containing an extended aromatic surface area, as well as the ability to mimic the function of protonated cytosine. The extended aromatic surface of this nucleobase should allow for more efficient stacking with neighboring nucleobases,[16,17] thereby increasing the stability of the triplexes. At the same time, the distribution of hydrogen bond donor and acceptor sites ensures specific interaction with the guanine(-cytosine) base(pair) (Fig. 1).

The Boc-PNA monomer containing the novel nucleobase, K, was available from the previously described (7-chloro-1,8-naphtphyridin-2(*1H*)-oxo-3-yl) acetic acid (2),[17] by the route depicted in Figure 2. The (1,8-naphtyridine-2,7(*1,8H*)-dioxo-3-yl)acetic acid (2) was prepared from the chloroderivative (1) by prolonged basic hydrolysis with refluxing aqueous NaOH (94%).[18] The

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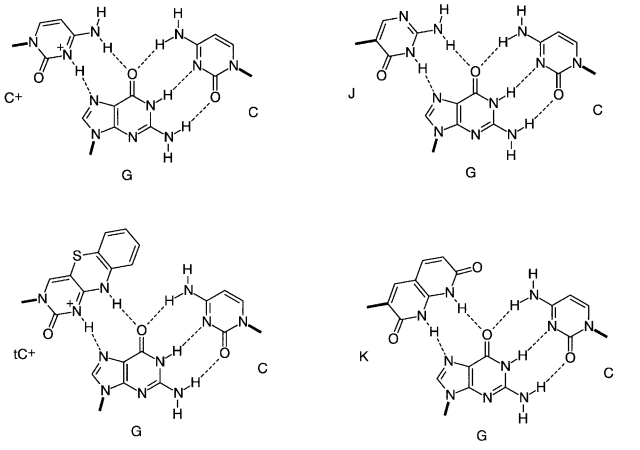

Figure 1. Hoogsteen type triplets formed by protonated cytosine (C+), protonated 3,5-diaza-4-oxophenothiazine (tC^+), pseudoisocytosine (J) and 1,8-naphthyridine-2,7(*1,8H*))-dione (K), respectively, with the G(-C) base(-pair). As shown, the diketo tautomer of the K nucleobase has the ability to mimic the function of protonated cytosine in the parallel, pyrimidine triple helix motif. At the same time, the increased surface area of the 1,8-naphthyridin-2,7(*1,8H*))-dione will allow for more efficient stacking within the third strand.

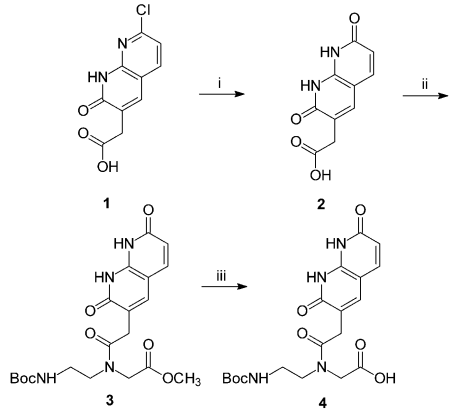

Figure 2. Synthetic route to the 1,8-naphthyridine-2,7(*1,8H*)-dione ring system was based on the synthesis of 7-chloro-1,8-naphthyridine-2(*1H*)-oxo-3-acetic acid, from which the corresponding 7-oxo compound (**2**) was obtained by basic hydrolysis. (i) NaOH (6 M, aqueous) at reflux, 19 h; (ii) Methyl *N*-(2-(*tert*-butyloxycarbonyl)aminoethyl)glycinate, HOAt and DCC in DMF, rt, overnight; (iii) NaOH (2 M, aqueous) in THF, rt, 15 min; then HCl (2 M, aqueous).

Boc-protected PNA monomer was prepared by condensation of (2) and methyl *N*-(2-(*tert*-butyloxycarbonyl)aminoethyl)glycinate[19] using 1-hydroxy-7-azabenzotriazole (HOAt) and, *N*,*N*'-dicyclohexylcarbodiimide (DCC) in DMF (46%),[20] followed by hydrolysis of the methyl ester with NaOH in THF (82%).[21]

PNA oligomerisation was performed according to the previously described procedure.[22,23] The Boc-PNA monomer, **4**, showed coupling efficiencies comparable to those found for the conventional PNA monomers. The recognition properties of the K heterocycle was evaluated as part of a bis-PNA[23] recognition systems, designed to recognize a single stranded oligonucleotide target by Watson–Crick and Hoogsteen base pairing. The thermal stability of the complex was analysed by thermal denaturation of the complex formed between the PNA oligomers and complementary oligodeoxyribonucleotides.[24] The thermal denaturation curves showed monophasic, well-defined transitions from which the T_m was obtained. The ability of the K base to recognise the guanine(cytosine) base (pair) was assessed by incorporation of the monomer, **4**, into the Hoogsteen strand of a bis-PNA in separated (PNA 4) as well as adjacent (PNA 8) positions (Table 1 and 2). The DNA target was chosen such that the three modified nucleobase units each faced a guanine(-cytosine) in the DNA (-PNA) target, and the remaining positions in the triplex consisted of conventional T·A-T triplets. The thermal stabilities of complexes formed between these bis-PNAs and the respective oligonucleotide targets was compared to those of the corresponding cytosine (C) (PNAs 1 and 5), pseudoisocytosine (J) (PNAs 2 and 6) and 3,5-diaza-4-oxophenothiazine (tC) (PNAs 3 and 7)[25] containing controls. Furthermore, the thermal stability was measured at pH 5.0, 7.0 and 9.0 to examine the dependence on pH.

In the bis-PNA sequence in which three modified nucleobases were incorporated into isolated positions, H-TXTXTXT-eg1-eg1-eg1-TCTCTCT-NH_2, the K containing PNA was superior to the other PNAs (C, J and tC) with respect to the thermal stability of the corresponding triplex complex at pH 7.0 (Table 1). The stabilisation per modification, arising from incorporation of the K nucleobase in the Hoogsteen strand, was 6.7 °C (relative to C) and 1.5 °C (relative to J). At pH 7.0, both cytosine and tC would be expected to be only partially protonated, and consequently not as effective hydrogen bond donors as the permanently protonated J and K nucleobases. However, at pH 5.0, electrostatic interactions between the positively charged cytosines (and tCs) and the negatively charged backbone of the target should favour binding. Even so, the K nucleobase was still comparable to cytosine (and to the tC nucleobase) at pH 5.0 (Table 1). In an isomeric sequence, in which the modified nucleobases where in adjacent positions, H-TTXXXTT-eg1-eg1-eg1-TTCCCTT-NH_2, both

Table 1. Thermal stability (T_m) of PNA•DNA-PNA complexes using a bis-PNA containing cytosine, pseudoisocytosine (J), phenothiazine (tC) or 1,8-naphthyridin-2,7(*1,8H*))-dione (K) respectively (in separated positions) hybridised to a DNA complement 5′-dCGCAGAGAGACGC-3′ containing the 7-mer target

bis-PNA sequence[a]	T_m (°C)[b]	ΔT_m (°C)
H-TCTCTCT-eg1-eg1-eg1-TCTCTCT-NH_2 (PNA 1)	42.0/50.5/78.0	−6.7/−5.2/+3.0
H-TJTJTJT-eg1-eg1-eg1-TCTCTCT-NH_2 (PNA 2)[c]	62.0/66.0/69.0	0/0/0
H-TtCTtCTtCT-eg1-eg1-eg1-TCTCTCT-NH_2 (PNA 3)[d]	41.0/42.0/70.0	−7.0/−8.0/+0.3
H-TKTKTKT-eg1-eg1-eg1-TCTCTCT-NH_2 (PNA 4)	55.0/70.5/77.5	−2.3/+1.5/+2.8

[a]Three units of 8-amino-3,6-dioxaoctanoic acid (eg1) connects the two anti-parallel PNA strands.
[b]The thermal stability of the complex with the DNA oligonucleotide 5′-AGAGAGA was determined at pH 9.0/7.0/5.0.
[c]*J* = pseudoisocytosine.
[d]tC = 3,5-diaza-4-oxophenothiazine.

Table 2. Thermal stability (T_m) of PNA·DNA-PNA complexes using a bis-PNA containing cytosine, pseudoisocytosine (J), phenothiazine (tC) or 1,8-naphthyridin-2,7(*1,8H*)-dione (K) respectively (in adjacent positions) hybridised to a DNA complement 5′-dCGCAAGG-GAACGC-3′ containing the 7-mer target

bis-PNA sequence[a]	T_m (°C)[b]	ΔT_m (°C)
H-TTCCCTT-eg1-eg1-eg1-TTCCCTT-NH_2 (PNA 5)	36.5/47.5/77.5	−6.5/−4.7/+3.0
H-TTJJJTT-eg1-eg1-eg1-TTCCCTT NH_2-(PNA 6)	56.0/61.5/68.5	0/0/0
H-TTtCtCtCTT-eg1-eg1-eg1-TTCCCTT-NH_2 (PNA 7)	34.5/44.5/74.5	−7.2/−5.3/+6.0
H-TTKKKTT-eg1-eg1-eg1-TTCCCTT-NH_2 (PNA 8)	65.5/75.5/76.5	+3.2/+4.7/+2.7

[a]Three units of 8-amino-3,6-dioxaoctanoic acid (eg1) connects the two anti-parallel PNA strands.
[b]The thermal stability of the complex with the DNA oligonucleotide 5′-AAGGGAA was determined at pH 9.0/7.0/5.0.

the cytosine and pseudoisocytosine containing triplexes exhibited slightly decreased stability (Table 2) relative to the situation of isolated positions (Table 1). However, the K nucleobase performed better in this sequence context, leading to a triplex of significantly higher stability relative to the sequence containing K in separated positions at pH 7 and 9. This is ascribed to the enhanced stacking overlap between neighboring K nucleobases. Relative to the cytosine and pseudoisocytosine containing sequences, the stabilization per substitution at pH 7.0 was 9.4 °C (relative to C) and 4.7 °C (relative to J) (Table 2).

These results show that the K base should be a useful improvement in the design of triplex forming PNAs, and possibly also of triplex forming oligonucleotides. Further investigations are underway to elucidate the potential of PNA oligomers containing the K nucleobase as tools for specific targeting of single and double stranded nucleic acids, as used in a range of biotechnology applications.[26,27]

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18. **(1,8-Naphthyridine-2,7(*1,8H*)-dion-3-yl)acetic acid (2)**. A suspension of (7-chloro-1,8-naphthyridin-2(*1H*)-on-3-yl)acetic acid [17] (4.00 g, 17.0 mmol) in NaOH (6 M, aqueous) (50 mL) was heated to reflux for 19 h and then allowed to cool to rt. The pH of the aqueous solution was adjusted to 7.0 by addition of HCl (concd, aqueous), the resulting precipitate collected by filtration and washed repeatedly with water to give the desired product (3.47 g, 94%) as a colourless powder (mp >250 °C). ^{1}H NMR (DMSO-d_6): δ 11.83 (br, 2H, NH), 7.81 (d, 8.8 Hz, 1H, arom), 7.69 (s, 1H, arom), 6.37 (d, 8.8 Hz, 1H, arom), 3.40 (s, 2H, CH_2). ^{13}C NMR (D_2O/NaOD): δ 184.03, 174.45, 173.44, 141.12, 139.79, 122.42, 113.63, 110.97, 41.92. FAB+MS (*m*/*z*): 175.92 (M−(COOH)+H$^+$, calcd for $C_9H_7N_2O_2$ 176.06).
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20. **Methyl *N*-((1,8-naphthyridine-2,7(*1,8H*)-dion-3-yl)acetyl)-*N*-(2-*tert*-butyloxycarbonyl)aminoethyl)glycinate (3)**. To a precooled solution of (1,8-naphtyridin-2,7(*1,8H*)-dion-3-yl)acetic acid (1.80 g, 8.38 mmol) and HOAt (1.34 g, 10.1 mmol) in DMF was added DCC (2.08 g, 10.1 mmol) and the mixture was stirred for 40 min at 0°C prior to addition of methyl *N*-(2-(*tert*-butyloxycarbonyl)aminoethyl)glycinate (2.22 g, 9.00 mmol). The mixture was stirred overnight at rt, evaporated in vacuo, redissolved in dichloromethane (100 mL) and DCU filtered off. The organic phase was washed with $NaHCO_3$ (satd, aqueous) (3×75 mL) and with brine (2×100 mL), dried over $MgSO_4$ and evaporated in vacuo. The crude product was purified on silica with AcOH/MeOH/CH_2Cl_2 (2/9/89 v/v/v) as the eluent, and fractions containing the product was pooled and evaporated in vacuo to yield the desired product (1.70 g, 46%) (mp 197–198°C). ^{1}H NMR (DMSO-d_6): δ 7.50 (7.55) (d, 8.8 Hz, 1H, arom), 7.45 (7.40) (s, 1H, arom), 6.93 (6.71) (m, 1H, NH), 5.99 (d, 8.8 Hz, 1H, arom), 4.04 (4.39) (s, 2H, CH_2), 3.63 (s, 3H, OCH_3), 3.47 (3.50) (s, 2H, CH_2), 3.32 (3.31) (m, 2H, CH_2), 3.16 (3.03) (m, 2H, CH_2), 1.35 (1.34) (s, 9H, CH_3(Boc)). ^{13}C NMR (DMSO-d_6): δ 171.54 (171.77), 170.17 (170.53), 165.89, 155.78, 148.79, 138.64, 136.48 (136.93), 118.01 (117.56), 111.67, 101.80, 77.90 (77.72), 51.75 (52.07), 48.21 (50.15), 47.59 (46.51), 38.10, 33.30 (33.88), 28.26. FAB$^+$MS (*m*/*z*): 435.1871 (M+H$^+$, calcd for $C_{20}H_{27}N_4O_7$ 435.1880).
21. *N*-((1,8-Naphthyridine-2,7(*1,8H*)-dion-3-yl)acetyl)-*N*-(2-(*tert*-butyloxycarbonyl)aminoethyl)glycine (**4**). To a precooled (0°C) solution of methyl *N*-((1,8-naphthyridin-2,7(*1,8H*)-dion-3-yl)acetyl)-*N*-(2-(*tert*-butyloxycarbonyl)aminoethyl)glycinate (1.70 g, 3.79 mmol) in THF (20 mL) was added NaOH (2M, aqueous)

(20 mL). This solution was stirred at rt for 15 min, additional water was added and the THF evaporated in vacuo. pH of the aqueous solution was adjusted to 3.0 by addition of HCl (2 M, aqueous) and the resulting precipitate washed with water (2×5 mL), collected by centrifugation and dried to yield the desired product (1.31 g, 82%) as a colourless powder (mp 195–97°C). ^{1}H NMR (DMSO-d_6): δ 11.90 (s br, 2H), 7.76 (7.83) (d, J = 8.8 Hz, 1H), 7.68 (7.61) (s, 1H), 6.89 (6.72) (m, 1H), 6.39 (6.38) (d, J = 8.8 Hz, 1H), 3.96 (4.21) (s, 2H), 3.53 (3.43) (s, 2H), 3.34 (m, 2H hidden by water signal), 3.16 (3.03) (m, 2H), 1.36 (1.35) (s, 9H). ^{13}C NMR (DMSO-d_6): δ 171.13 (171.48), 170.82 (170.46), 163.37, 162.51, 147.30, 139.14 (139.33), 137.25 (137.52), 122.17, 109.00, 105.90, 78.01, 48.08 (50.38), 47.60 (46.76), 38.41 (38.02), 33.43 (33.14), 28.27. FAB + MS (m/z): 421.1723 ($M + H^+$, calcd for $C_{19}H_{25}N_4O_7$ 421.1723), 443.1543 ($M + Na^+$, calcd for $C_{19}H_{24}N_4O_7Na^+$ 443.1543).

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