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Synthesis and evaluation of a conformationally constrained pyridazinone PNA-monomer for recognition of thymine in triple-helix structures

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Abstract—A novel conformationally constrained pyridazinone E^{ag}-base PNA-monomer **2** capable of binding thymine in a triplex motif was designed and synthesised. A bis-PNA with the E^{ag}-base incorporated in the Hoogsteen strand was hybridised with a complementary DNA. Thermal stability studies revealed an increase in T_m (4.3 °C per mod.) compared to a no-base unit, but showed no improvement over a previously described unconstrained analogue (E, **1**). Surprisingly, no significant difference was found in the thermodynamic parameters (ΔH°, ΔS° and ΔG°) for PNA–DNA triplex formation involving **2** or the unconstrained analogue **1**.
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Sequence specific triplex targeting of double stranded DNA in mixed purine/pyrimidine targets remains a challenge in bioorganic chemistry. Due to the asymmetric nature of the triple helix only a purine strand is recognised through Hoogsteen base pairing.[1] To expand the triplex recognition repertoire of nucleic acids for gene targeting purposes, novel nucleobases that recognise the pyrimidine part of the Watson–Crick base pairs C-(G) and T-(A) are required. Nucleobases that to some extent recognise cytosine have been described.[1,2] Recent approaches to the recognition of thymine relies on hydrogen bond contacts to both bases of the A–T pair.[3,4] The previously described E-base PNA-monomer **1**[5] (Fig. 1) is able to recognise T when incorporated into the Hoogsteen strand of a bis-PNA. To reach the O4 of thymine in the major groove this monomer contains a linker that is two atoms longer compared to conventional PNA-monomers. Also, an extra nitrogen was build into the base in order to minimise steric clash with the 5-methyl group of thymine.[5] However, the binding affinity is far from optimal which might partly be due to excessive flexibility of the backbone-'nucleobase' linker and/or steric clash with the 5-methyl group of thymine. The latter possibility is based on the observation that E-base PNAs bind somewhat stronger to uracil- than to thymine-containing targets.[5] In order to address the issue of flexibility, we have now synthesised a new analogue **2** (Fig. 1). This analogue contains a double bond that should significantly restrict the flexibility in the linker, and thereby reduce the added loss of entropy associated with triplex formation.

Furthermore, the double bond might yield enthalpic stabilisation through stacking interactions with neighbouring base pairs.

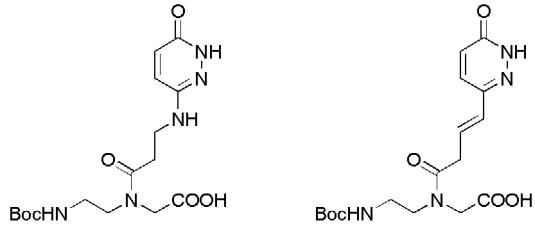

Figure 1. Structures of E-base PNA monomer (**1**) and E^{ag}-base PNA monomer (**2**).

Keywords: DNA recognitition; Triplex; Nucleobase; Peptide nucleic acid (PNA).
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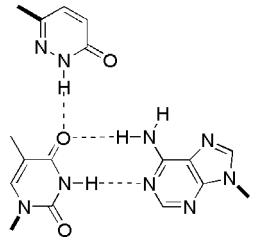
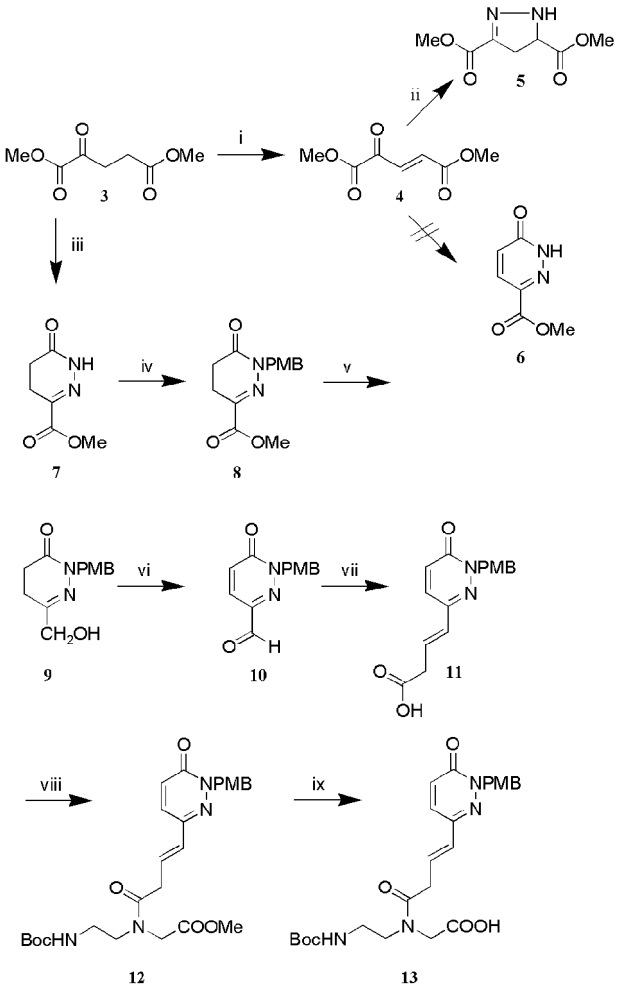


Figure 2. Proposed structure of the E^{ag}/E·T-A triplet.

The specific recognition of thymine (uracil) is expected to be identical for both monomers, through an NH hydrogen bond to the 4-oxo group of thymine (Fig. 2).

The structural differences of the linker in **1** and **2** lead to the implementation of a distinct synthetic strategy. In order to prepare the conformationally constrained PNA-monomer **13** (a protected **2**) commercially available dimethyl 2-oxoglutarate (**3**) was chosen as starting material. Several approaches where examined before the successful route depicted in Scheme 1 was found. Thus a direct route via **4** to a suitable substituted pyridazinone **6** failed, the main product being the pyrazoline **5**[6] instead. In the successful route **3** was first transformed to the known dihydropyridazinone **7**[7] using hydrazine with acid catalysis in refluxing methanol. The next steps were initially tried without a *N*-1 protecting group, but due to very low solubility of later compounds, especially the unprotected acid corresponding to compound **11**, this strategy was abandoned. To improve solubility, compound **7** was reacted with 4-methoxybenzyl chloride and sodium hydride in DMF to obtain the PMB protected product **8**. Reduction of **8** using sodium borohydride in a refluxing mixture of THF and methanol gave the primary alcohol **9**. Treatment of **9** with activated manganese(IV) oxide in refluxing toluene afforded a tandem reaction where the primary alcohol was first oxidised to the aldehyde and next an oxidative aromatisation took place to give compound **10**. This reduction–oxidation strategy to obtain the aldehyde was chosen since an earlier attempt with aromatisation using $CuCl_2$ followed by Dibal-H reduction of the ester was unsuccessful. The next step, a Wittig reaction between aldehyde **10** and 2-carboxyethyltriphenylphosphonium bromide,[8] took place in a 1:1 mixture of THF and DMSO in the presence of sodium hydride. Only the desired E-isomer **11** was obtained, as verified by a typical *trans* CH-CH coupling constant of 16.3 Hz. Condensation of **11** with *N*-(2-Bocaminoethyl)-glycine methyl ester[9] using DCC and DHbtOH afforded the ester **12**. Finally, hydrolysis of the ester with NaOH in methanol resulted in the monomer **13**.[10,11]

The monomer **13** could be incorporated into PNA oligomers under standard solid phase conditions,[13] and removal of the *p*-methoxybenzyl protecting group took place under these conditions. The E^{ag} base was evaluated in a bis-PNA[14] recognition system designed to recognise a single stranded DNA oligonucleotide target by Watson–Crick and Hoogsteen base pairing (Fig. 3). The E^{ag} base was incorporated in the Hoogsteen strand of a bis-PNA at two positions opposite an adenine in



Scheme 1. Reagents, conditions and yields: (i) ref 12 (2 steps); (ii) NH_2NH_2, AcOH, MeOH, reflux, 12 h (42%, 3 steps); (iii) NH_2NH_2, AcOH, MeOH, reflux, 12 h (92%); (iv) 4-methoxybenzyl chloride, NaH, DMF, 0 °C, 1 h then rt, 12 h (80%); (v) $NaBH_4$, THF, MeOH, reflux, 1 h (58%); (vi) activated MnO_2, toluene, reflux, 24 h (30%); (vii) $Ph_3P^+(CH_2)_2COOH\ Br^-$, NaH, THF/DMSO, 0 °C, 1 h then rt, 4 h (42%); (viii) methyl *N*-(2-Boc-aminoethyl) glycinate, DhbtOH, DCC, DMF, 0 °C, 1.5 h then rt, 12 h (50%); (ix) 2M NaOH, MeOH, 0 °C, 2.5 h (46%).

the Watson–Crick strand (Table 1) and therefore facing thymine in the DNA target. The remaining positions consisted of conventional T·A-T and J·G-C triplets. The ability of the E^{ag}-base to recognise thymine was evaluated by incorporation of a no-base PNA monomer[5] or the original E-base monomer in the same position for comparison. The thermal stability of the complexes formed between these bis-PNAs and complementary DNA was analysed by thermal denaturation. The temperature versus absorbance profiles showed monophasic, well defined transitions, from which the T_m were obtained. The results summarised in Table 1 show significant stabilisation when the E^{ag}-base rather than a no-base position faces thymine ($\Delta T_m = +4.3$ °C per mod.), but the triplex is less stable than when using the original E-base ($\Delta T_m = -1.5$ °C per mod.).

The nucleobase discrimination of E^{ag} was studied by introduction of a double C-mismatch as presented in

Table 1. Thermal stability (T_m) of PNA·DNA–PNA complexes using a bis-PNA containing two no-bases, E-bases or E^{ag}-bases respectively hybridised to a DNA complement 5′-dCGCAGATAGTAAACGC-3′ containing the 10-mer target

Bis-PNA sequence[a]	T_m (°C)[c]
H-TJT**aeg(Ac)**TJ**aeg(Ac)**TTTeg1eg1eg1TTTACTATCT-NH_2[b]	47.5
H-TJT**E^{ag}**TJ**E^{ag}**TTTeg1eg1eg1TTTACTATCT-NH_2	56.1
H-TJT**ETJE**-TTTeg1eg1eg1TTTACTATCT-NH_2	59.1

[a] Three units of 8-amino-3,6-dioxaoctanoic acid (eg1) connect the two antiparallel 10-mer PNA strands. Pseudoisocytosine (J) was used instead of protonated cytosine in the Hoogsteen strand of the bis-PNA.
[b] aeg (Ac) is *N*-acetyl-*N*-(2-aminoethyl) glycine, a no-base PNA unit.
[c] All *T*m measurements in this paper were performed in 100 mM NaCl, 0.1 mM EDTA, 10 mM Na_2HPO_4, pH 7.0 on a Cary 300Bio spectrophotometer at a heating rate of 0.5 °C/min.

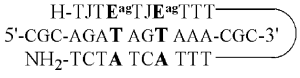


Figure 3. Structure of the bis-PNA–DNA complex.

Tables 2 and 3. These results demonstrate a similar ΔT_m discrimination against cytosine as the original E base (15 and 14 °C, respectively). However, the recognition of U was different. Whereas the E-base preferred binding to U over T ($\Delta T_m = +3.5$ °C per mod.), the new E^{ag}-base showed a minor preference of binding to T compared to U ($\Delta T_m = +0.5$ °C). This suggests the absence of significant steric clash between the E^{ag}-base and the 5-methyl group of thymine.

To gain further insight into the binding behaviour, the thermal stabilities were measured at different concentrations in order to obtain thermodynamic data for the bis-PNA–DNA complexes according to the following equation.[15]

$$1/T_m = (R/\Delta H^0)\ln C_{tot} + (\Delta S^0 - R\ln 4)/\Delta H^0 \quad (1)$$

Table 2. Effect of target base changes on PNA·DNA–PNA complexes using the following bis PNA: H-TJT**E^{ag}**TJ**E^{ag}**TTT-eg1eg1eg1-TTTACTATCT-NH_2

Oligonucleotide target sequence	*T*m (°C)	ΔT_m/mod. (°C)
5′-dCGCAGA**T**AGT**A**AACGC-3′	56.1	
5′-dCGCAGA**U**AGU**A**AACGC-3′	55.0	−0.5
5′-dCGCAGA**C**AGC**A**AACGC-3′	26.0	−15.0

Table 3. Effect of target base changes on PNA·DNA–PNA complexes using the following bis PNA: H-TJT**ETJE**TTT-eg1eg1eg1-TTTACTATCT-NH_2

Oligonucleotide target sequence	T_m (°C)	ΔT_m/mod. (°C)
5′-dCGCAGA**T**AGT**A**AACGC-3′	59.1	
5′-dCGCAGA**U**AGU**A**AACGC-3′	66.2	+3.5
5′-dCGCAGA**C**AGC**A**AACGC-3′	31.1	−14.0

Table 4. Thermodynamic data for the bis-PNA–DNA complexes with E^{ag} and E respectively. The data are based on three independent experiments each including measurement of T_m at ten different complex concentrations (SEM is indicated in parenthesis). ΔG^0 was calculated from the ΔH^0 and ΔS^0 values obtained from the $1/T_m$ versus $\ln C_{tot}$ plot

	H-TJT**E^{ag}**TJ**E^{ag}**TTTeg1eg1 eg1TTTACTATCT-NH_2	H-TJT**ETJE**TTTeg1eg1 eg1TTTACTATCT-NH_2
ΔH^o	−706 (±34) kJ/mol	−722 (±96) kJ/mol
ΔS^o	−2037 (±102) J/mol	−2058 (±288) J/mol
ΔG^o	−74.0 (±2.0) kJ/mol	−83.4 (±6.6) kJ/mol

As expected from the T_m data, these results (Table 4) showed slightly lower free energy for the bis-PNA complex with the E-base, but most surprisingly no significant differences between the two monomers in the relative values of the thermodynamics parameters (ΔH^0, ΔS^0 or ΔG^0) could be detected. In particular the entropy loss was identical. Therefore, introduction of the more rigid E^{ag}-base did not result in an entropic advantage.

The results reported herein could be used as guidelines for design of future analogues. Contrary to expectation PNA clamps containing the new monomer **2** performed poorer in DNA hybridisation assays than the fully flexible analogue, and as concluded from thermodynamic measurements, it is especially noteworthy that the entropy loss upon triplex formation is virtually identical for the two PNAs. Therefore, the higher flexibility of the E-base cannot be the reason for the relatively weak binding to thymine. This could indicate that local flexibility has only little influence on overall binding thermodynamics of oligonucleotide hybridisation, and that the entropy term is predominantly determined by overall flexibility of the oligomer in combination with contributions from changes in solvation.

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10. Part of this work has been presented as a poster at the XV International Round Table conference on 'Nucleosides, Nucleotides and Nucleic Acids', Leuven, Belgium, September 2002: Olsen, A. G.; Dahl, O.; Nielsen, P. E. *Nucleosides, Nucleotides and Nucleic Acids* **2003**, *22*, 1331.
11. Selected data for new compounds: 1-(4-methoxybenzyl)-6-oxo-1,4,5,6,-tetrahydropyridazine-3-carboxylic acid methyl ester (**8**): ^{1}H NMR ($CDCl_3$) δ 7.22 (d, 2H, J = 6.6 Hz, PMB), 6.75 (d, 2H, J = 6.6 Hz, PMB), 4.86 (s, 2H, CH_2-benzyl), 3.78 (s, 3H), 3.69 (s, 3H) (2×CH_3O), 2.77 (t, 2H, J = 8.8 Hz, CH_2), 2.45 (t, 2H, J = 8.8 Hz, CH_2). FAB-MS m/z 277 ($M+H^+$). 1-(4-methoxybenzyl)-6-oxo-1,4,5,6,-tetrahydropyridazine-3-methanol (**9**): ^{1}H NMR (CD_3OD) δ 7.27 (d, 2H, J = 6.6 Hz, PMB), 6.87 (d, 2H, J = 6.6 Hz, PMB), 4.86 (s, 2H, CH_2-benzyl), 4.20 (s, 2H, CH_2OH), 3.79 (s, 3H, CH_3O), 2.63 (m, 2H, CH_2), 2.53 (m, 2H, CH_2). TOF-HRMS-ES+ m/z calcd for $C_{13}H_{17}N_2O_3$, 249.1239, found: 249.1232. 1-(4-Methoxybenzyl)-6-oxo-1,6-dihydropyridazine-3-carbaldehyde (**10**): ^{1}H NMR ($CDCl_3$) δ 9.66 (s, 1H, CHO), 7.62 (d, 1H, J = 9.5 Hz, arom), 7.35 (d, 2H, J = 6.6 Hz, PMB), 6.87 (d, 1H, J = 9.5 Hz, arom), 6.78 (d, 2H, J = 6.6 Hz, PMB), 5.26 (s, 2H, CH_2-benzyl), 3.70 (s, 3H, CH_3O). FAB-MS m/z 245 ($M+H^+$). *E*-4-[1-(4-Methoxybenzyl)-6-oxo-1,6-dihydropyridazin-3-yl]-but-3-enoic acid (**11**): ^{1}H NMR (CD_3OD) δ 7.72 (d, 1H, J = 9.7 Hz, arom), 7.34 (d, 2H, J = 6.8 Hz, PMB), 6.95 (d, 1H, J = 9.7 Hz, arom), 6.88 (d, 2H, J = 6.8 Hz, PMB), 6.56 (dt, 1H, J = 16.3, 6.8 Hz, CH=), 6.47 (d, 1H, J = 16.3 Hz, CH=), 5.24 (s, 2H, CH_2-benzyl), 3.77 (s, 3H, CH_3O), 3.31 (d, 2H, J = 6.8, 1.1 Hz, CH_2). TOF-HRMS-ES+ m/z calcd for $C_{16}H_{17}N_2O_4$: 301.1188, found: 301.1201. *N*-(2-Bocaminoethyl)-*N*-{4-[1-(4-methoxybenzyl)-6-oxo-1,6-dihydropyridazin-3-yl]-but-3-enoyl} glycine methyl ester (**12**): This compound exist as two rotamers; chemical shifts for the minor rotamer are given in brackets. ^{1}H NMR (CD_3OD) δ 7.73 (7.72) (d, 1H, J 9.7 Hz, arom), 7.30 (d, 2H, J = 6.8 Hz, PMB), 6.94 (d, 1H, J = 9.5 Hz, arom), 6.85 (d, 2H, J = 6.8 Hz, PMB), 6.50 (m, 2H, 2×CH=), 5.22 (s, 2H, CH_2-benzyl), (4.29), 4.11 (s, 2H, CH_2), 3.76 (s, 3H, CH_3O), (3.71), (s, 3H, CH_3) 3.52 (t, 2H, J = 6.2 Hz, CH_2), 3.47 (d, 2H, J = 5.1 Hz, CH_2), 3.25 (3.19), (t, 2H, J = 6.2 Hz, CH_2), 1.42, (1.40) (s, 9H, Boc). TOF-MS-ES+ m/z 515.3 ($M+H^+$). *N*-(2-Bocaminoethyl)-*N*-{4-[1-(4-methoxybenzyl)-6-oxo-1,6-dihydropyridazin-3-yl]-but-3-enoyl} glycine (**13**): This compound exists as two rotamers; chemical shifts for the minor rotamer are given in brackets. ^{1}H NMR (DMSO-d_6) δ (7.77), 7.76 (d, 1H, J = 9.7 Hz, arom), 7.27–7.18 (m, 1H, BocNH), 7.24 (d, 2H, J = 8.8 Hz, PMB), (6.93), 6.92 (d, 1H, J = 9.7 Hz, arom), 6.88 (d, 2H, J = 8.8 Hz, PMB), 6.53 (m, 1H, CH=), (6.34) 6.25 (d, 1H, J = 16.3 Hz, CH=), 5.12 (s, 2H, CH_2-benzyl), 3.71 (s, 3H, CH_3O), (3.61), 3.60 (s, 2H, CH_2), 3.30 (m, 2H, CH_2), 3.18 (d, 2H, J = 6.8 Hz, CH_2), 3.04 (m, 2H, CH_2), (1.36) 1.34 (s, 9H, Boc), TOF-HRMS-ES+ m/z calcd for $C_{25}H_{33}N_4O_7$: 501.2349, found: 501.2348.
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