ORGANIC LETTERS

2002
Vol. 4, No. 8
1283−1286

2,6-Diaminopurine in TNA: Effect on Duplex Stabilities and on the Efficiency of Template-Controlled Ligations[1]

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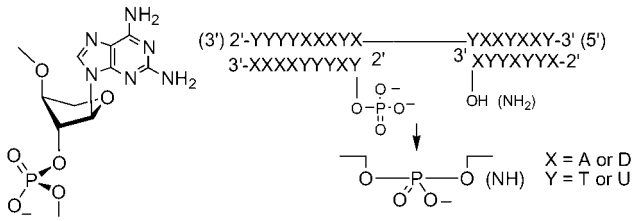
Received January 16, 2002

ABSTRACT



Replacement of adenine by 2,6-diaminopurine—two nucleobases to be considered equivalent from an etiological point of view—strongly enhances the stability of TNA/TNA, TNA/RNA, or TNA/DNA duplexes and efficiently accelerates template-directed ligation of TNA ligands.

The constitutional simplicity and remarkable base-pairing properties of TNA (α-threofuranosyl-(3′→2′)-oligonucleotides) raise the question of a possible role of threose-derived informational oligomers in the origin of RNA.[2−4] Such a proposition calls for a comprehensive experimental investigation of the chemical properties of TNA and related oligomer systems containing threose-derived backbones.[2,3] With this in mind, we have extended our investigations on the base-pairing properties of TNA to sequences that contain 2,6-diaminopurine as a nucleobase in place of adenine and have tested the propensity of TNA and RNA sequences to act as templates for the ligation of TNA ligands. Here we report that replacement of adenine by 2,6-diaminopurine in TNA[5]—not unexpectedly—strongly enhances the stability of TNA/TNA, TNA/RNA, and TNA/DNA duplexes and efficiently accelerates template-directed ligation reactions.

2,6-Diaminopurine's capacity to stand for adenine in Watson−Crick base pairs, and to enhance duplex stability by virtue of its three (instead of two) hydrogen bonds to uracil or thymine, has been demonstrated in a variety of studies for a number of oligonucleotide systems. [6−9]

N^9-α-L-Threofuranosyl nucleoside of 2,6-diaminopurine in its N^2,N^6-protected form (**4**), whose structure is supported by an X-ray analysis,[10] was prepared from the L-threofuranose derivative (**1**) and 2,6-diaminopurine-N^2,N^6-dibenzoate[11] (**2**), closely following the procedures described earlier[2] for

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(5) From an etiological point of view, 2,6-diaminopurine is not to be considered an unnaturally complex nucleobase; it can assemble from the same type of elementary building blocks as guanine can.

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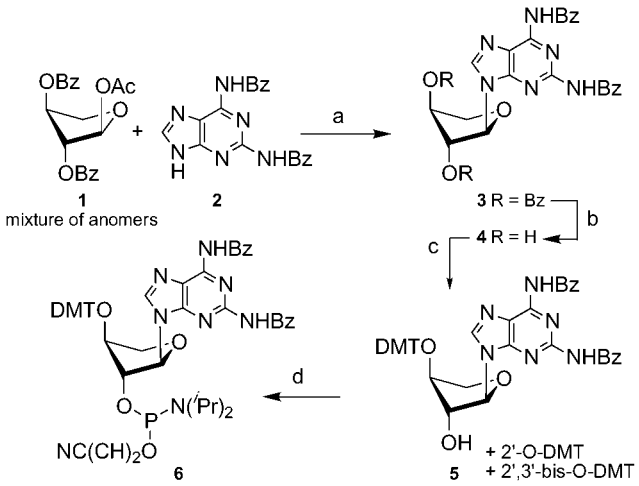
10.1021/ol020016p CCC: $22.00
Published on Web 03/16/2002

Scheme 1[a]



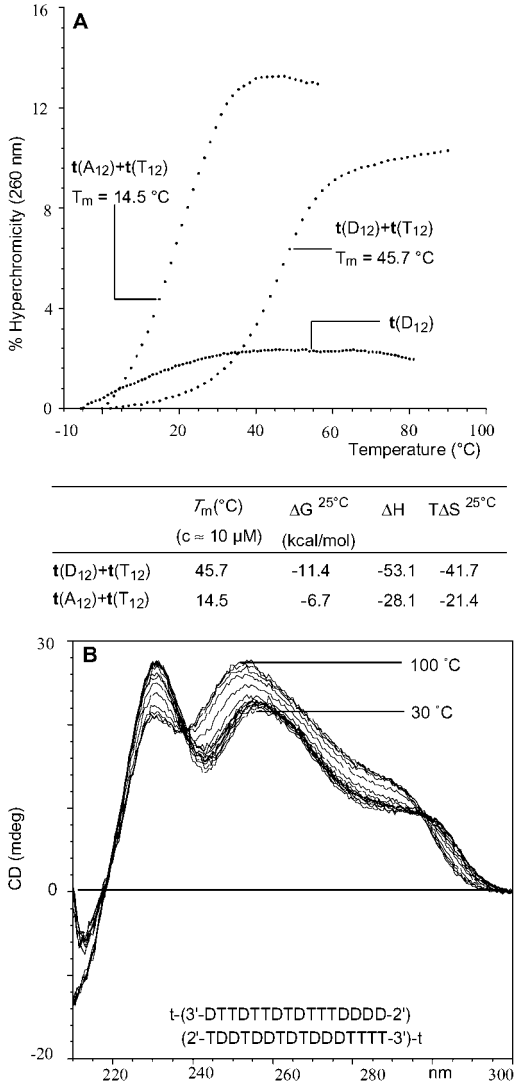
[a] Numbers in parentheses denote molar equivalents referring to starting compound or ratios. Numbers before these parentheses denote concentrations in M. (a) 0.16 M (1.2 molar equiv) of **2**, 0.43 (3.1) BSA, 0.18 (1.3) TMSOTf, CH_3CN, 65 °C, 3 h; 59%; (b) 2 M NaOH, THF/MeOH/H_2O (5:4:1), 0 °C, 15 min; 87%; (c) 0.16 M (1.3) DMTCl, CH_2Cl_2/DMF (1:1), 0.7 (5.9) 2,6-lutidine, 0.18 (1.5) AgOTf, rt, 4 h, then (0.2) DMTCl, (0.2) AgOTf, rt, 20 h; 3′-O-DMT 40%, 2′-O-DMT 20%, 2′,3′-bis-O-DMT 6%; (d) 0.98 (5.7) chloro(cyanoethoxy)(diisopropylamino)phosphine, 0.53 (3.1) N(*i*-Pr)$_2$Et, CH_2Cl_2, rt, 2 h; 94%. X-ray structure analysis of **4**: torsion angles: O−C(2′)-C(3′)-O, 77.7°; O−C(1′)-N(9)−C(4), 166.8°.

other TNA nucleosides (Scheme 1). TNA sequences containing 2,6-diaminopurine (= D) required for our studies were synthesized on solid support according to the previously reported procedure[2] using intermediate **6**.[12]

The duplex t(A_{12})/t(T_{12}), exemplifying the purine/pyrimidine sequence motif that is known to be associated with weak pairing in TNA,[13] raises its T_m value from 14 to 45 °C by (A → D) replacement (standard conditions, see Figure 1).

A similar enhancement of duplex stability is observed in TNA/TNA, TNA/RNA, and TNA/DNA duplexes composed of the hexadecamer sequence $A_4T_3ATAT_2AT_2A$ and its antiparallel complement (the RNA sequence containing A and U, the DNA sequence A and T) (Table 1). The duplex containing D in both of its TNA strands is more stable than the duplexes from all other combinations (Table 1). Those data of Table 1 which refer to differences induced by (A → D) replacement correspond to an average enhancement of duplex stability per single (A → D) replacement of about −0.7 kcal/mol DDG (25 °C).[14]

Our experiments on the effect of (A → D) replacements on the efficiency of template-directed ligations[15,16] of TNA ligands focused on the same hexadecamer duplex which we used for the stability studies, taking 3′(or 5′)-TA_2TA_2-$TATA_3T_4$-2′(or 3′) as the template sequence and using a water-soluble carbodiimide as the activating agent[17] (Table



	T_m(°C) (c ≈ 10 μM)	ΔG 25°C (kcal/mol)	ΔH	TΔS 25°C
t(D_{12})+t(T_{12})	45.7	-11.4	-53.1	-41.7
t(A_{12})+t(T_{12})	14.5	-6.7	-28.1	-21.4

Figure 1. (**A**) UV-spectroscopic T_m curves (heating) of the duplexes t(T_{12})·t(A_{12}) and t(T_{12})·t(D_{12}) (no hysteresis of cooling curves). T_m values are derived from the maxima of the first-derivative curve (software Kaleidagraph). Thermodynamic data are derived from plots of T_m^{-1} with versus ln (*c*);[25] experimental error estimated in ΔH values ±5%. (**B**) Temperature-dependent CD curves of the duplex formed between t-(3′-DTTDTTDTDTTTD-DDD-2′) and its complementary sequence ($c \approx 5 + 5$ μM, temperature range 30−100 °C). All measurements were made in 1.0 M NaCl, 10 mM NaH_2PO_4, 0.1 mM Na_2EDTA, pH 7.0.

(10) Carried out by Raj K. Chadha, TSRI. Crystallographic data for the structure has been deposited with the Cambridge Crystallographic Data Center as deposition No. CCDC 175169. Copies of the data can be obtained, free of charge, on application to the CCDC, 12 Union Road, Cambridge, CB12 1EZ UK (fax + 44 (1233) 336 0333; e-mail deposit@ccdc.cam.ac.uk).

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(12) All compounds were characterized by ^1H and ^{13}C NMR and mass spectral data after purification by column chromatography on silica gel. A full experimental account will be published in *Helv. Chim. Acta*. Oligonucleotides were synthesized on an Expedite 8909 Nucleic Acid Synthesizer (Perseptive Biosystems), purified by HPLC (ion exchange column SAX 1000-8 (Macherey-Nagel)) up to a minimal purity of 95% and shown to have the expected molecular mass by MALDI-TOF MS (Supporting Information). Ligand sequences containing a 2′-phosphate group were made using a DMTO-ethylsulfonyl derivatized-CPG solid support (see Bolli, M.; Micura, R.; Pitsch, S.; Eschenmoser, A. *Helv. Chim. Acta* **1997**, *80*, 1901; formula in footnote 5). Ligand sequences containing 3′-amino threofuranosyl adenine as the end group were synthesized using the required building block described in ref 3. RNA oligonucleotides were purchased from Xeragon Inc. and DNA(A,T) oligonucleotides from Operon Technologies Inc.

Table 1. T_m Values ($c \approx 5 + 5$ μM) and Thermodynamic Data of the Duplexes Formed by Cross-Pairing between the Hexadecamer Base Sequences Indicated Bearing a TNA, RNA, or DNA Backbone[a]

(or 5') 3'–XXXXYYYXYXYYXYYX–2' (or 3')
(or 3') 2'–YYYYXXXYXYXXYXXY–3' (or 5')

T_m (°C) / $\Delta G_{25\,°C}$ / ΔH / $T\Delta S$ (Kcal/mol)	TNA X = D Y = T	TNA A T	RNA A U	DNA A T
TNA X = D Y = T	79 -24.6 -107.3 -82.7	67 -20.3 -98.9 -78.6	74 -25.8 -125.2 -99.4	56 -20.1 -83.6 -63.5
TNA A T	67 -17.8 -79.0 -61.2	56 * -14.5 -73.7 -59.2	57 * -17.3 -93.9 -76.6	47 * -13.3 -77.3 -64.0
RNA A U	65 -21.6 -120.4 -98.8	52 * -15.8 -95.2 -79.4	59 * -20.0 -131.9 -111.9	44 * -12.3 -73.9 -61.6
DNA A T	49 -15.0 -91.6 -76.7	41 * -11.0 -57.0 -46.0	43 * -13.7 -98.0 -84.3	48 * -16.8 -129.2 -112.4

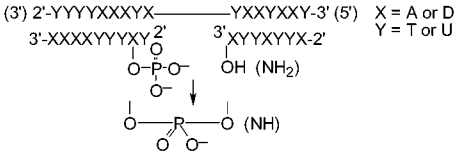
[a] The color of the acronyms relates to oligonucleotide sequence of the same color in the duplex. The labels 3′ and 2′ indicate strand orientation in TNA sequences; for RNA and DNA sequences, the corresponding labels are 5′ and 3′. Sequences are written in the 3′ → 2′ or 5′ → 3′ Ddirection. T_m values in the shaded diagonal refer to intrasystem pairing; all others to intersystem-crosspairing. For conditions, see caption of Figure 1.

2). Ligation rate enhancements by (A → D) replacement have been demonstrated for the natural oligonucleotide series.[18] We also addressed questions referring to the sequence fidelity to be expected in TNA ligation, as well as to the degree by which in the TNA series phosphate-to-phosphoramidate ligations are more efficient[19] than phosphate-to-phosphodiester ligations. Examples of intersystem-template-controlled ligations involving other artificial base-pairing systems have been recently described.[20]

The formation of a phosphodiester bond between the unmodified TNA ligands t(A_4T_3AT)-2′-phosphate and t(AT_2-AT_2A)-3′-OH (see Table 2 and Figure 2 in the Supporting Information) driven by EDC[17] under the control of the unmodified TNA(A,T)-hexadecamer sequence mentioned above as a template is, under the conditions chosen (see Table 2), a slow and rather unproductive reaction.[21] However, the same process with ligands containing the nucleobase D instead of A produces the corresponding ligation product in essentially quantitative yields within hours. The same pattern holds for ligations involving the TNA template containing D instead of A. Furthermore, a similar acceleration due to (A → D) replacement is observed when ligations leading to a phosphoramidate bond are compared. Phosphate-to-phosphoramidate ligation of the D-containing TNA ligands is so fast that side reactions do not discernibly interfere, and the ligation products are obtained in essentially quantitative yield (HPLC) within minutes. Whereas (A → D) replacement in the TNA ligands strongly accelerates ligation in either phosphodiester or phosphoramidite formation, such is not observed when A is replaced by D in the TNA template sequence; both templates give rise to comparable ligation efficiencies. Remarkably, an RNA template sequence (containing A and U) is as efficient in bringing about ligation of the TNA ligands as the TNA templates themselves. However, DNA as a template fails to induce phosphate-to-phosphodiester ligations to any discernible extent; yet it brings about an essentially quantitative phosphate-to-phosphoramidate ligation when the TNA ligands contain D in place of A.

Figure 3 summarizes experiments carried out to assess sequence fidelity. The medium-fast phosphate-to-phosphodiester ligation of the D-containing TNA ligands was chosen for comparing combinations that involve a single mismatch in the 9-mer ligand and/or a single mismatch (in two

Table 2. TNA(A,T)-, TNA(D,T)-, RNA-(A,U)-, and DNA(A,T)-Templated Ligations of TNA Ligands: Effects of (A → D) Replacement in Templates and TNA Ligands and of (OH → NH_2) End-Group Replacement in the Ligand−Nucleophile on the Rate of Ligation[a]



ligands		templates					
		TNA(A,T)		TNA(D,T)		RNA(A,U)	DNA(A,T)
TNA-2′-OPO_3^{2-}	TNA HO -3′−H_2N−						
(A,T)	HO-	4 h	19%		14%	30%	4 h
(D,T)	HO-	1 h	57%		51%	53%	4 h
		4 h	>95%	5 h	>95%	4 h >95%	
(A,T)	H_2N-	10 min	59%		52%	43%	
		30 min	>95%	33 min	95%	50 min >95%	4 h 37%
(D,T)	H_2N-	10 min	94%		80%	60%	
		13 min	>95%	20 min	>95%	45 min >95%	5 h >95%

[a] Percentages refer to the yield of ligation product formed after the reaction times indicated as monitored by ion-exchange HPLC (see Figure 2 in the Supporting Information). Ligations were carried out in 0.1 M aqueous HEPES buffer with 0.1 mM template, 0.2 mM ligand (each), 0.2 M EDC, pH 7.5, rt All ligation products have been identified by MALDI-TOF mass spectroscopy and most of them, in addition by co-injection with authentic material. Test experiments involving the (A,T)-TNA-3′-NH_2 and (A,T)-TNA-2′-phosphate ligands in the *absence* of any template did not show any discernible ligation. Note that the nucleoside unit that bears the 3′-amino end group has A instead of D.

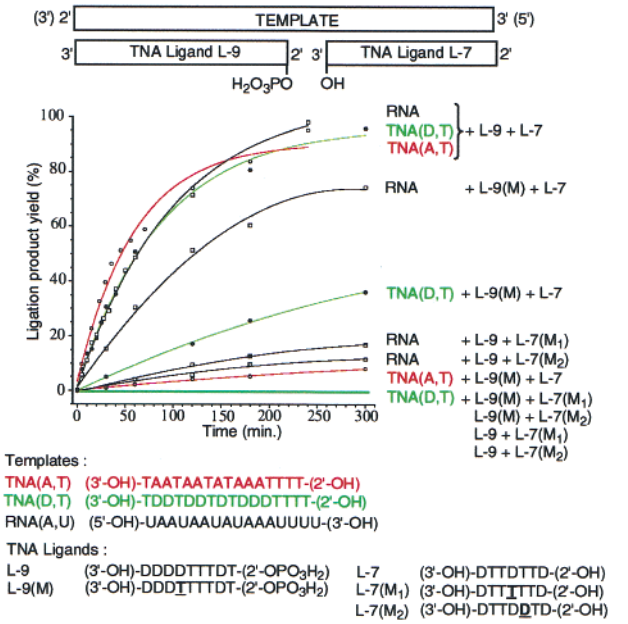


Figure 3. Effect of mismatches (M) in TNA 9-mer and 7-mer ligands on the efficiency of phosphate-to-phosphodiester ligation of TNA ligands (containing D and T) mediated by templates of the TNA and RNA series. T_m values of ligation triplexes (phosphodiester series). L-9 + L-7 + template (5 + 5 + 5 μM): TNA-(A,T), 22 °C; TNA(D,T), 37 °C. RNA(A,U): 32 °C. L-9(M) + L-7 + template: TNA(A,T), <0 °C; TNA(D,T), <0 °C; RNA-(A,U), 13 °C. For conditions of ligations, see caption of Table 2.

versions) in the 7-mer ligand. Expectedly, a single mismatch in the 9-mer ligand turns out to be less harmful to the ligation than one in the 7-mer ligand, the relative rates depending, however, on the template. Offering both monomismatched ligands concomitantly to any of the three templates does not lead to ligation at all. This amounts to a remarkably high sensitivity of the ligation to the presence of mismatches in the TNA series.[22]

Under the conditions where the RNA template (containing A and U) can direct the phosphate-to-phosphodiester ligation of the TNA ligands even somewhat more efficiently than the two TNA templates themselves (Figure 2 in the Supporting Information), the latter do not induce the phosphate-to-phosphodiester ligation of the RNA ligands r(AAAAU-UUAU) and r-5′-phosphate-(AUUAUUA). To keep this result in perspective, it must be pointed out that under otherwise identical conditions the RNA template was found not to induce ligation of the two RNA ligands to any discernible extent either.[23] The requisites (sequence lengths, nucleobases, type of activation and conditions) for a transcription of TNA into RNA sequences remain to be explored.

Among the observed TNA ligations, relative efficiencies qualitatively parallel the stability of complexes between ligands, or between ligation products and templates. Thus, the remarkably different response of the three templates toward the single mismatch in the nonamer ligand reflects itself in the T_m values of the corresponding ternary ligation complexes (see caption of Figure 3). Expectedly, there are exceptions, such as the observations according to which (A → D) replacement in the TNA ligands accelerates ligations whereas (A → D) replacement in the TNA template does not (Table 2)[24] or that DNA, as compared to RNA, seems unproportionately inefficient as template relative to its cross-pairing capability with TNA. A decisive factor for the efficiency of intersystem template-controlled ligations must be the constellational positioning of the two ligands' reaction districts by the template.[16] Within the studies presented here, this factor seems to express itself most prominently in the observed asymmetry between the efficiency of RNA-templated TNA ligation and that of TNA-templated RNA ligations.

Acknowledgment. This work was supported by the Skaggs Research Foundation. X.W. is a Skaggs Postdoctoral Fellow. G.D. thanks the Skaggs Research foundation and la Universidad Nacional Autónoma de México for fellowship support.

Supporting Information Available: X-ray structure of **4**, MALDI-TOF mass data of sequences, HPLC traces and relative rate curves of selected ligations, and T_m values of ligand−template, product−template, and ligand(mismatch)−template complexes. This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) Model considerations point to poor interstrand base stacking in the (weakly base pairing) homopurine−homopyrimidine duplexes of TNA as opposed to the (much more stable) duplexes in which purines and pyrimidines are alternating (compare the low hyperchromicity in $t(A_{12})$/$t(T_{12})$ and its D-analogue in Figure 1, as compared to a hyperchromicity of 35−40% for TNA duplexes $(AT)_8$, $(AU)_8$, or $(TA)_8$).

(14) Stability rise increments are expected to depend on sequences and are not expected to remain the same with increasing the number of (A → D) replacements. See: Sági, J.; Szakanyi, E.; Vorlícková, M.; Kypr, J. *J. Biomol. Struct. Dyn.* **1996**, *13*, 1035.

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(21) Slow ligation rates allow side reactions (due to large excess of the carbodiimide activation agent) to hamper product formation.

(22) For recent studies on the fidelity of template-controlled ligations see, for examle, Kenneth, J.; Ellington, A. D. *Chem. Biol.* **1997**, *4*, 595. Bolli, M.; Micura, R.; Pitsch, S.; Eschenmoser, A. *Helv. Chim. Acta* **1997**, *80*, 1901. Mattes, A.; Seitz, O. *Chem. Commun.* **2001**, 2050.

(23) For the inefficiency of chemical ligation of RNA ligands on DNA templates, see the following: Dolinnaya, N. G.; Sokolova, N. I.; Ashirbekova, D. T.; Shabarova, Z. A. *Nucleic Acids Res.* **1991**, *19*, 3067.

(24) Self-pairing of template strands are expected to contribute to this phenomenon. T_m values (c ≈ 5 μM, 1 M NaCl) of template strands: TNA-(A,T), <5 °C; TNA(D,T), 30 °C.

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