# Synthesis and Structural Characterization of PNA-DNA Quadruplex-Forming Chimeras

Veronica Esposito,[a] Antonio Randazzo,[a] Anna Messere,[b] Aldo Galeone,[a] Luigi Petraccone,[c] Concetta Giancola,[c] Gennaro Piccialli,[a] and Luciano Mayol*[a]

**Keywords:** Chimeras / DNA / Quadruplexes / Synthesis

The synthesis and structural characterization of four PNA-DNA chimeras – $^{5'}$TGGG$^{3'}$-t (**C1**), $^{5'}$TGG$^{3'}$-gt (**C2**), t-$^{5'}$GGGT$^{3'}$ (**C3**) and tg-$^{5'}$GGT$^{3'}$ (**C4**), where lower and upper case letters indicate PNA and DNA residues, respectively – are reported. The oligomers were synthesized through the use of commercially available Bhoc/Fmoc PNA monomers and 3′- or 5′-phosphoramidite nucleosides as building blocks. All the molecules were studied by $^1$H NMR, CD and UV spectroscopy and by molecular modelling. The CD spectra and NMR spectroscopic data of **C1** and **C3** are typical of quadruplexes involving four parallel strands. Moreover, the UV melting profiles indicate that their thermal stabilities are quite similar to that observed for the reference quadruplex [d(TGGGT)]$_4$. The $^1$H NMR spectra of **C2** and **C4** show that these oligomers are not able to fold into a single, well defined species.


## Introduction

PNA is a versatile molecule, characterized by a pseudopeptide backbone that mimics the phosphodiester counterpart in nucleic acids. It has been shown to form several types of stable double-stranded structures (PNA·DNA,[1] PNA·RNA[1] and PNA·PNA[2]) as well as triple helices [DNA·(PNA)$_2$,[3] RNA·(PNA)$_2$,[4] PNA·(DNA)$_2$,[5] and (PNA)$_3$ [6]]. These properties have suggested potential applications of PNA as antisense and antigene agents.[4,7,8] Furthermore, very recently, the formation of a (PNA)$_2$·(DNA)$_2$ hybrid quadruplex has been reported,[9] confirming the ability of PNA to mimic DNA even in more complex multistranded structures. However, poor water solubility, together with the inability to activate RNase H in PNA·RNA heteroduplexes,[4] rather limit these potential applications. Substantial improvements in the biological, chemical and physical properties of PNA have been achieved through the construction of PNA-DNA chimeras[10] in which a DNA strand is bound to a PNA moiety. PNA-DNA chimeras possess interesting biological properties as antisense[11] agents and also as decoys against NF-kB transcription factors.[12] Furthermore, some types of PNA-DNA chimeras have also been shown to form triple helix structures.[13,14]

In an effort to expand the versatility of these molecules, we have been investigating the ability of PNA-DNA chimeras to form quadruplex structures. In fact, the presence of a PNA tract should enhance the exonuclease stability and hence the biological activity of these structures, the relevance of which has been dramatically increased by their presence in telomeric sequences and in several biologically active aptamers, as well as by finding of a number of quadruplex-binding proteins.[15]

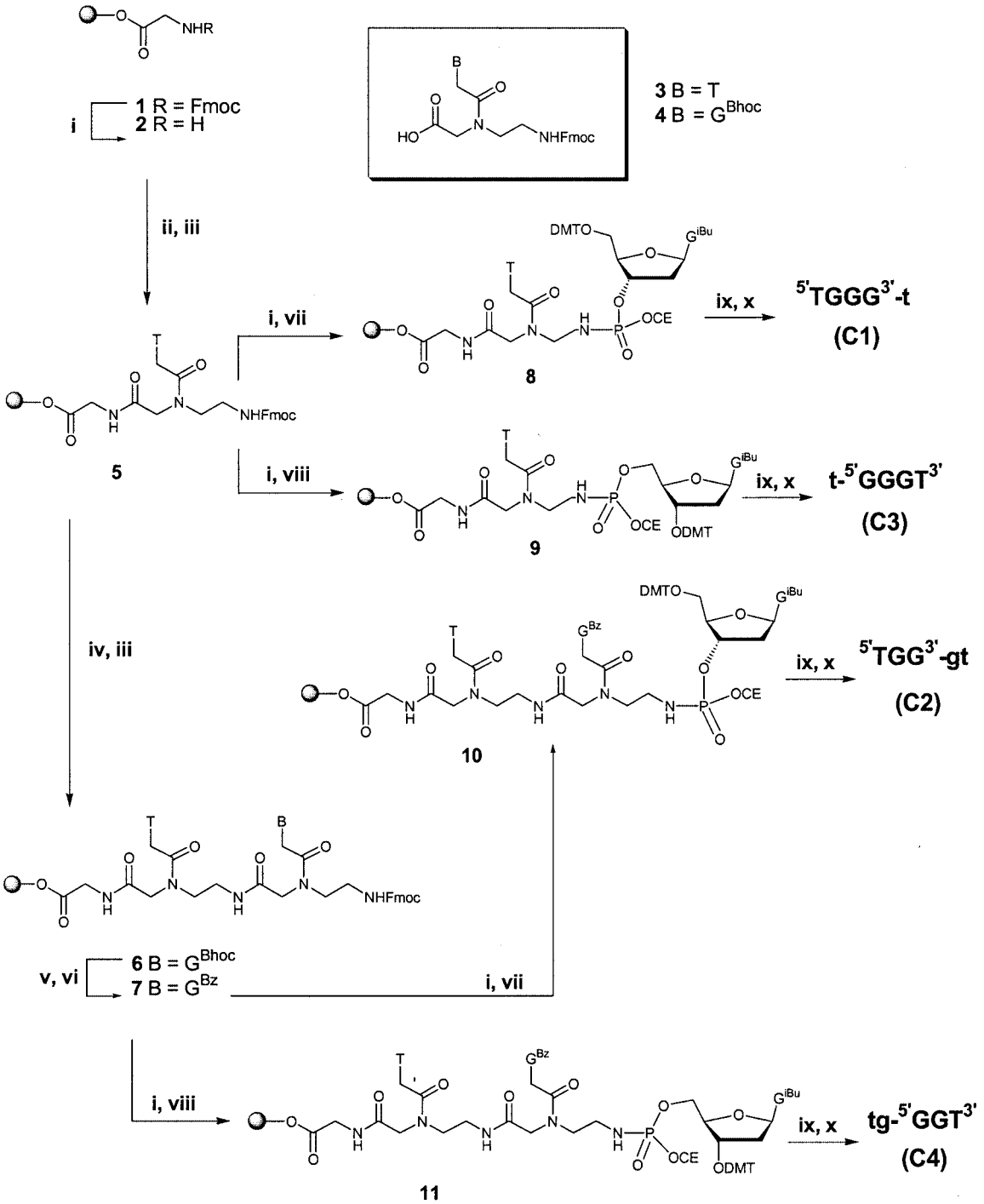
## Results and Discussion

The oligonucleotide d(TGGGT) is known to form a parallel quadruplex [d(TGGGT)]$_4$, the structure of which has been already investigated by $^1$H NMR spectroscopy.[16] We therefore proposed to replace some nucleotide residues at the 3′- or 5′-end of this sequence with the corresponding PNA counterparts, in order to evaluate the effects on the formation of quadruplex complexes. The following chimeras were therefore prepared: $^{5'}$TGGG$^{3'}$-t (**C1**), $^{5'}$TGG$^{3'}$-gt (**C2**), t-$^{5'}$GGGT$^{3'}$ (**C3**) and tg-$^{5'}$GGT$^{3'}$ (**C4**), where lower and upper case letters indicate PNA and DNA residues, respectively. **C1** and **C2** were synthesized by a recently reported solid-phase strategy[17] based on the use of commer-

[a] Dipartimento di Chimica delle Sostanze Naturali, Università degli Studi di Napoli "Federico II",
Via D. Montesano 49, 80131 Napoli, Italy
Fax: (internat.) + 39-081/678552
E-mail: mayoll@unina.it
[b] Dipartimento di Scienze Ambientali, Seconda Università di Napoli,
Via Vivaldi 43, 81100 Caserta, Italy
[c] Dipartimento di Chimica, Università degli Studi di Napoli "Federico II",
Via Cintia, 80126, Napoli, Italy

  DOI: 10.1002/ejoc.200300213 

cially available Bhoc/Fmoc PNA monomers (**3**, **4**) and 3′-phosphoramidite nucleosides as building blocks (Scheme 1). This synthetic approach has been further implemented here by the use of 5′-phosphoramidite nucleosides for easy synthesis of PNA-DNA chimeras containing 5′-phosphoramidate bonds (**C3** and **C4**) (Scheme 1). Both synthetic approaches use a Tentagel-OH resin functionalized with an N-Fmoc glycine residue (**1**). After deprotection of the amino group, the first PNA unit **3** was attached to support **2**, to give **5**. After the removal of the Fmoc protecting group, support **5** was treated with 5′- (or 3′)-DMT-amino-protected guanosine-3′-(or 5′)-phosphoramidite, thus furnishing support **8** (or **9**), which in turn, after completion of the synthesis of DNA tract, afforded the chimeric pentamer **C1** (or **C3**). Alternatively, Fmoc-deprotected support **5**, after treatment with Bhoc/Fmoc guanine PNA monomer **4**,




Scheme 1. Reagents: (i) piperidine/DMF, 1:4 v/v; (ii) **3**, HATU, DIPEA, DMF/$CH_3CN$, 1:1 v/v; (iii) $Ac_2O$/piperidine, 2:3 v/v (capping step); (iv) **4**, HATU, DIPEA, DMF/$CH_3CN$, 1:1 v/v; (v) TFA in DCM 75% w/w; (vi) BzCl/pyridine, 3:7 v/v; (vii) coupling with 3′-phosphoramidite monomer; (viii) coupling with 5′-phosphoramidite monomer; (ix) DNA chain assembly on the automated synthesiser; (x) concd. $NH_4OH$

provided support **6**. It has been demonstrated[17] that the Bhoc group is unstable under detritylation conditions (DCA/DCM) and its final removal under acidic conditions may induce depurination on dG nucleotides. For these reasons, **6** was converted into **7** by replacement of the Bhoc guanine protecting group with a benzoyl (Bz) group, which is completely stable under the conditions of the standard phosphoramidite technique.

As in the case of **5**, treatment of **7** with 5′-(or 3′)-DMT-amino-protected guanosine-3′-(or 5′)-phosphoramidite furnished support **10** (or **11**), the direct precursor of the chimeric pentamer **C2** (or **C4**). Thus, in **C1** and **C2**, the 3′-phosphoester end of the DNA tract is linked through a 3′-phosphoramidate bond to the N-terminal of the PNA moiety [(C)-PNA-(N)-p-3′-DNA-5′], whereas **C3** and **C4** are linked to the N-terminal of the PNA moiety through a 5′-phosphoramidate bond [(C)-PNA-(N)-p-5′-DNA-3′]. No significant difference in the coupling yields of 3′- and 5′-phosphoramidite nucleosides with amino groups of PNA was observed in these reactions. After detachment from the solid support and deprotection, the crude oligomers were purified by HPLC, desalted and analysed by ESI mass spectrometry. Furthermore, the quadruplex complexes were investigated by $^1$H NMR, CD and UV spectroscopy, and by molecular modelling.

In a preliminary structural analysis of quadruplex-forming oligonucleotides, $^1$H NMR spectroscopic data both from exchangeable and from non-exchangeable protons can provide useful information. For example, the number of slowly exchanging imino protons can substantiate the strand stoichiometry, the number of G quartets and the symmetry of the quadruplex structure. Furthermore, the range of proton resonance frequencies may indicate the presence of Watson−Crick or Hoogsteen base-pairing as well as the presence of exchange-protected imino protons from unpaired residues. In particular, imino proton chemical shifts observed in the $\delta = 10.5-12$ ppm range are indicative of guanine NH/O hydrogen bonds, typically found in Hoogsteen arrangement of the G quartets.[18,19]

In our case, the $^1$H NMR spectra of both $^{5'}$TGGG$^{3'}$-t (**C1**) (Figure 1, A) and t-$^{5'}$GGGT$^{3'}$ (**C3**), in a buffer stabilizing quadruplex structures (see Exp. Sect.), show the presence of five signals belonging to three guanine H8 and two thymine H6 protons in the aromatic region. It is interesting to note that the proton signals of PNA bases are shifted upfield in comparison with those of the corresponding DNA residues. Moreover, three well defined singlets were detected in the $\delta = 11-12$ ppm region, thus suggesting the presence of imino protons involved in Hoogsteen hydrogen bonds of G tetrads.

These data are consistent with the formation of G-quadruplex structures containing three G-tetrads and possessing fourfold symmetry with all strands parallel to each other. The exchange rates of the imino protons with solvent for **C1** and **C3** were qualitatively estimated by partial drying of the samples in water and their reconstitution in $D_2O$. Periodic examination of the imino proton signals showed that they exchange very slowly, in agreement with what has been observed for other quadruplex structures.[20] Proton signals for **C1** and **C3** have been partially assigned on the basis of NOESY and TOCSY data obtained at 500 MHz (T = 300 K). The NOEs between H2′/H2′′ and H6/H8 are initially used for peak assignment (Figure 1, B). The observation of an unbroken path of NOE connectivities along the strands, in contrast to what is observed for antiparallel quadruplex structures, suggests that the backbone conformations of both **C1** and **C3** are similar to that of regular parallel quadruplex DNA.[21] The relative intensities of NOEs observed between G H8 and ribose H2′, in comparison with the NOEs observed between G H8 and H1′, indicates that the glycosidic torsion angles in all G residues are in *anti* conformations and the polarity connectivities (G H8 to ribose protons on the 5′ side only) are indicative of right handed helices, as would be expected for a parallel quadruplex.[22−24]

Further evidence of the formation of parallel quadruplexes for **C1** and **C3** could be obtained from circular dichroism data. The resulting CD spectra are very similar to that produced by the canonical $[d(TGGGT)]_4$, with the presence of maximum and minimum Cotton effects at 263 nm and 243 nm, respectively, which is typical of

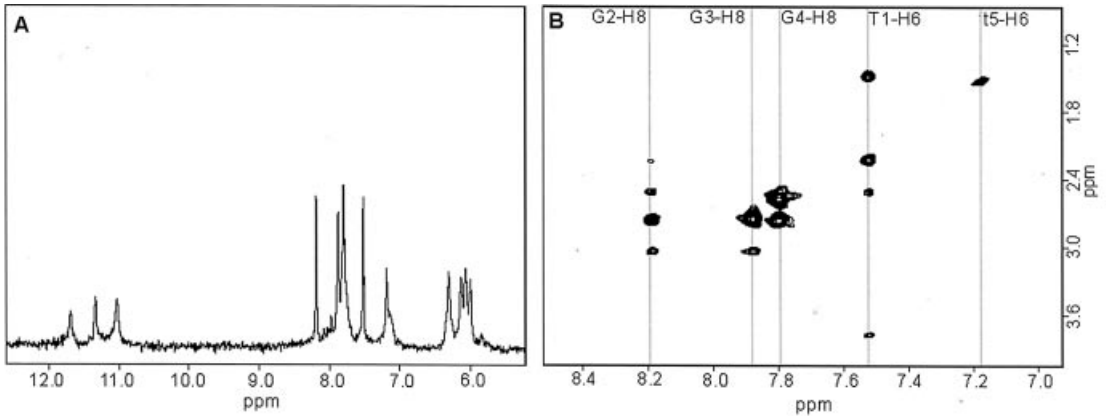



Figure 1. Aromatic and imino proton regions of the 500-MHz $^1$H-NMR spectrum (A) and H2′/H2′′ to base region of the phase-sensitive NOESY spectrum (B) of **C1**

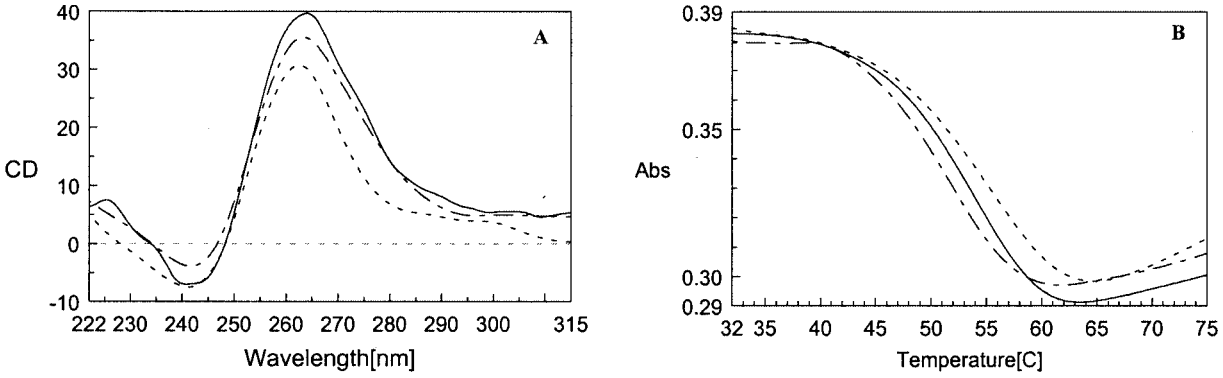



Figure 2. CD spectra (A) and melting profiles (B) of $[d(TGGGT)]_4$ (−), **C1** (− −) and **C3** (− − -)

quadruplexes involving four parallel strands[16] (Figure 2, A).

In the case of $^{5'}TGG^{3'}$-gt (**C2**) and tg-$^{5'}GGT^{3'}$ (**C4**), the $^1H$ NMR spectra are very complex and not attributable to well defined and stable single structures. In order to simplify these spectra, by eliminating signals due to aggregation phenomena sometimes present in solution, we recorded $^1H$ NMR spectra at higher temperatures of up to 323 K. In this range, unfortunately, no spectra presented signals attributable to single and well defined quadruplex structures, while at 323 K only the resonances corresponding to the single strands were present. In order to estimate the effects on thermal stability of the substitution of DNA residues with PNA units, the quadruplexes **C1**−C**4** were further analysed by UV thermal denaturation experiments. The melting profiles of **C1**, **C3** and the corresponding canonical $[d(TGGGT)]_4$, recorded at 295 nm,[25] give well-shaped sigmoid curves (Figure 2, B). The $T_m$ values, calculated as the maxima of the first derivative plots of absorbance vs. temperature, are listed in Table 1. These data show that thermal stabilities of both **C1** and **C3** are quite similar to that observed for the reference structure $[d(TGGGT)]_4$, thus indicating that both the PNA and the glycine moieties, as well as the different PNA-DNA junctions − namely (C)-PNA-(N)-p-3′-DNA-5′ (**C1**) and (C)-PNA-(N)-p-5′-DNA-3′ (**C3**) − do not affect the quadruplex stability. As expected, no significant melting curves could be obtained for chimeras **C2** and **C4**.

In order to explore the ability of the PNA tract of the strand to participate in the quadruplex formation, molecular mechanic calculations were performed on **C1**, **C2**, **C3** and **C4**.

The general appearance of all structures clearly suggests that the PNA tract is perfectly able to adapt its backbone to the quadruplex structure motif. In particular, all the bases of the PNA domains retain the correct orientation to form the Hoogsteen H-bonds after energy minimisation and, in the same way, the stacking interactions are also retained. Furthermore, in all four models, the PNA backbone is able to adopt a correct geometry without causing any distortion in the PNA-DNA junction region and, consequently, the Hoogsteen hydrogen bonds in the oligonucleotide tract of the quadruplex are also preserved. It is interesting to note in all models that glycine residues at the 3′-end or at the 5′-end of the quadruplexes form a "cage", which could be a possible coordination site for a cation. Molecular models of the quadruplexes formed by $^{5'}TGGG^{3'}$-t (**C1**) and t-$^{5'}GGGT^{3'}$ (**C3**) are shown in Figure 3.

Table 1. Melting temperatures of the quadruplexes formed by chimeras **C1** and **C3** and $[d(TG_3T)]_4$

| Compound | $T_m$ (°C) |
|---|---|
| $[d(TG_3T)]_4$ | 55.1 |
| $[d(TG_3$-t$)]_4$ (**C1**) | 52.9 |
| $[d($t-$G_3T)]_4$ (**C3**) | 54.9 |

## Conclusion

We report the usage of 5′-phosphoramidite nucleosides for easy preparation of PNA-DNA chimeras containing 5′-phosphoramidate bonds. We then explored the capability of PNA-DNA chimeras to form quadruplex structures. Results from NMR and CD clearly indicate that chimeras $^{5'}TGGG$-t$^{3'}$ (**C1**) and t-$^{5'}GGGT^{3'}$ (**C3**) adopt quadruple helical structures. The CD spectra of **C1** and **C3** show bands characteristic of quadruplexes and are similar to that of $[d(TGGGT)]_4$, which exists as a tetramolecular system. In particular, NMR spectra show that the modified quadruplexes possess fourfold symmetry with all strands parallel to each other and all DNA nucleosides in *anti* conformations.

Furthermore, as indicated by UV experiments, the presence of the PNA moiety at the edge of the quadruplexes, as well as the different PNA-DNA junctions − namely (C)-PNA-(N)-p-3′-DNA-5′ (**C1**) and (C)-PNA-(N)-p-5′-DNA-3′ (**C3**) − do not affect the thermal stability of the molecules.

On the other hand, the $^{5'}TGG$-gt$^{3'}$ (**C2**) and tg-$^{5'}GGT^{3'}$ (**C4**) sequences do not form well defined structures. This is probably due to the flexibility of the PNA backbone, which renders quadruplex formation entropically unfavourable on increasing the number of PNA units in the DNA-PNA strands.

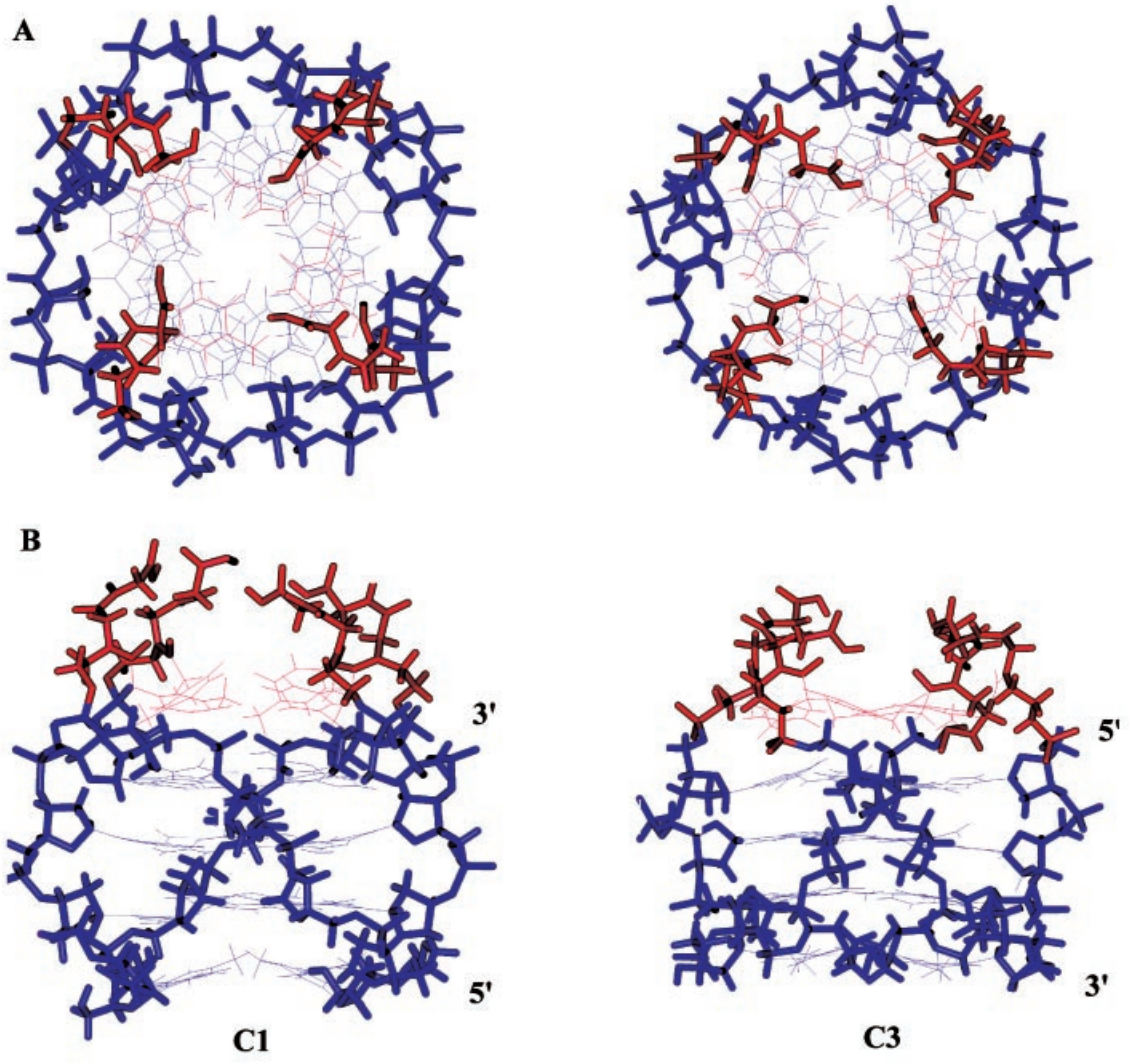



Figure 3. Molecular models of quadruplex structures **C1** and **C3**; the PNA tract is in red; A: top view, B: front view

However, it is interesting to note that PNA-DNA chimeras with only one PNA unit at the 3′-ends are still 25 times more stable in human serum than the corresponding unmodified oligodeoxynucleotides.[26] Therefore, substitution of a DNA residue with a PNA one in biologically active quadruplex-forming oligonucleotides (i.e. aptamers) could, in principle, improve the activity of those molecules. The effects of such modifications on structural and biological stability is currently under investigation in our laboratories.

## Experimental Section

**General Remarks:** The following abbreviations are used throughout the test: benzhydryloxycarbonyl (Bhoc), fluorenylmethoxycarbonyl (Fmoc), 4,4′-dimethoxytrityl (DMT), isobutyl (*i*Bu), dichloroacetic acid (DCA), dichloromethane (DCM), *O*-7-azabenzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU), diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), *N*,*N*-dimethylformamide (DMF), *N*,*N*′-dicyclohexylcarbodiimide (DCCI), *N*-hydroxybenzotriazole (HOBt), benzoyl chloride (BzCl), cyanoethyl (CE).

The Tentagel-OH resin was purchased from Novabiochem. The solid support functionalization and PNA tract assembly were carried out in a short glass column (12 cm length, 1 cm i.d.) with a sintered glass filter, a stopcock and a cap. The oligonucleotides were synthesized on a Millipore Cyclon Plus DNA synthesiser by solid-phase β-cyanoethyl phosphoramidite chemistry, cleaved from the support and purified by standard procedures. HPLC purifications were carried out with a Waters 515 Pump and a Waters 2487 UV detector. NMR spectra were recorded with a Bruker AMX 500 spectrometer (500.13 MHz for $^1$H) at 300 K. The solvent signals were used as internal standards, which were related to TMS with $\delta = 4.734$ ppm ($H_2O$ and HDO).

ESI mass spectra were recorded with a API 2000 (Applied Biosystem) machine used in negative mode. UV measurement and thermal denaturation experiments were carried out with a Jasco V-530 UV spectrophotometer equipped with a Jasco 505T temperature controller unit, with detection at $\lambda = 295$ nm. CD spectra were obtained with a Jasco 715 circular dichroism spectrophotometer.

**Functionalization of the Resin. Supports 1 and 2:** Fmoc-Gly-OH (476 mg, 1.60 mmol) and DCCI (330 mg, 1.60 mmol) were dissolved in DMF (6.0 mL). After 10 min at room temp. the resulting mixture and HOBt (216 mg, 1.60 mmol) were added to Tentagel-OH resin (572 mg, 0.28 mmol/g of hydroxy groups), previously washed with DMF. The mixture was kept at room temp. with shaking for 24 h. The support was filtered and washed with DMF and $Et_2O$, and then dried under reduced pressure, thus providing support **1** (0.25 mmol/g, 89% coupling yield). Support **1** was washed with pyridine and then treated with acetic anhydride in pyridine (6.0 mL, 2:3, v/v, 1 h, room temp.) to block the unchanged hydroxy groups. The level of incorporation of the glycine residue was calculated by quantitative UV measurements (300 nm) of the fluorene derivative released by piperidine/DMF treatment on **1** (1:4, v/v, 3 × 10 min) to afford support **2**.

**Synthesis of PNA Tract (General Procedure). Supports 5−7:** Support **5** was obtained by washing of support **2** (500 mg, 0.125 mmol) with DMF and then by leaving it in contact with a DMF/$CH_3CN$ solution (6.0 mL, 1:1 v/v) of the thymine PNA monomer (**3**, 506 mg, 1 mmol) in the presence of HATU (380 mg, 1 mmol) and DIPEA (259 μL, 1.5 mmol) for 1 h at room temp. with shaking. The coupling yields, measured as for support **1**, were 90%. A capping procedure was carried out on support **5**, after washing with DMF, by the addition of acetic anhydride in pyridine (2:3, v/v, 1 h, room temp.). The total amount of support **5** was divided into two parts (250 mg each). The first was used for the elongation of the DNA tracts (in order to obtain **C1** and **C3**; see below), whereas the second portion, after removal of Fmoc group, was treated with a solution of guanine PNA monomer (**4**, 334 mg, 0.45 mmol) in N-methylpyrrolidone (4.0 mL) containing few drops of DMF, in the presence of HATU (171 mg, 0.45 mmol) and DIPEA (117 μL, 0.67 mmol) with shaking for 1 h at room temp., to yield support **6** (0.20 mmol/g, 90% coupling yield). Support **6** (200 mg, 0.04 mmol) was washed with DCM and then treated with a solution of TFA in DCM (75% w/w, 6.0 mL) for 1 h at room temp. After exhaustive washings with DCM and pyridine, a solution of benzoyl chloride in pyridine (6.0 mL, 3:7 v/v, 6 h, room temp.) was added as acylating mixture. The resin was then washed with pyridine, DMF and $Et_2O$ and dried under reduced pressure, thus furnishing support **7**.

**Synthesis of DNA Tract (General Procedure). Products C1−C4:** Support **5** was divided into two fractions (100 mg each), and these were independently washed with DMF and then left in contact with a solution of piperidine in DMF (20%, 3 × 10 min, room temp.). After washings with DMF and $CH_3CN$, a phosphoramidite chemistry-based coupling step with guanosine 5′-*O*-DMT-3′-*O*-(2-cyanoethyl)phosphoramidite or guanosine 3′-*O*-DMT-5′-*O*-(2-cyanoethyl)phosphoramidite building blocks was performed on each support's fraction. These coupling steps, which gave supports **8** and **9**, respectively (80−85% coupling yield), were carried out with solutions of the amidite units (50 mg/mL) in $CH_3CN$ in the presence of tetrazole standard solution and with use of longer reaction times (20 min) than standard and were repeated twice. The oxidation step (20 min, room temp.) was performed by use of the oxidizing standard solution ($I_2$/pyridine/$H_2O$). ODN chain assembly was performed on each supports on an automated DNA synthesiser by the standard phosphoramidite procedure with final DMT removal. As before, 5′-*O*-DMT-3′-*O*-(2-cyanoethyl)phosphoramidite and 3′-*O*-DMT-5′-*O*-(2-cyanoethyl)phosphoramidite nucleoside building blocks were used on supports **8** and **9**, respectively, to complete the synthesis of the DNA tract. Two sequences were assembled (**C1** and **C3**), with observed coupling yields always being greater than 98%. Support **7** was divided into two fractions (100 mg each) and, after Fmoc removal, was treated with 3′- or 5′-phosphoramidite guanosine building blocks as described above, to afford supports **10** and **11** respectively. Two sequences were assembled (**C2** and **C4**) with observed coupling yields always greater than 98%.

**HPLC Purification of Oligomers C1−C4:** Oligomers **C1−C4** were detached from the support and deprotected by treatment with concd. aqueous ammonia at 55 °C for 12 h. The combined filtrates and washings were concentrated under reduced pressure, redissolved in $H_2O$, and analysed and purified by HPLC on a Nucleogel SAX column (Macherey−Nagel, 1000−8/46); buffer A: 20 mM $KH_2PO_4$ aq. solution, pH 7.0, containing 20% (v/v) $CH_3CN$; buffer B: 1 M KCl, 20 mM $KH_2PO_4$ aq. solution, pH 7.0, containing 20% (v/v) $CH_3CN$; a linear gradient from 0 to 100% B in 30 min, flow rate 1 mL/min., were used. The isolated oligomers (retention times: **C1** = 11.9 min; **C2** = 6.1 min; **C3** = 11.9 min; **C4** = 6.1 min) were collected and successively desalted on Sep-Pak columns (C18). The isolated oligomers proved to be more than 97% pure (NMR) and were further analysed by ESI-MS: **C1** and **C3**: $m/z$ = 1617 [M − H]$^-$; **C2** and **C4**: $m/z$ = 1579 [M − H]$^-$.

**Nuclear Magnetic Resonance:** NMR samples were prepared at concentrations of approximately 0.5 mM, in 0.5 mL ($H_2O/D_2O$, 9:1 v:v) buffer solution containing 10 mM potassium phosphate, 70 mM KCl, 0.2 m M EDTA, pH 7.0. For $D_2O$ experiments, the $H_2O$ was replaced with $D_2O$ by drying down of the sample, lyophilization and redissolution in $D_2O$ alone. 1D proton spectra of samples in $H_2O$ were recorded with the use of pulsed-field gradient watergate[27] for $H_2O$ suppression. Phase-sensitive NOESY spectra[28] were recorded with mixing times of 50, 100 and 200 ms ($T$ = 300 K). Pulsed-field gradient watergate was used for NOESY spectra in $H_2O$. TOCSY spectra[29] with mixing times of 120 ms were recorded with $D_2O$ solutions. NOESY and TOCSY were recorded with use of the TPPI[30] procedure for quadrature detection. In all 2D experiments the time domain data consisted of 2048 complex points in $t_2$ and 400−512 fids in $t_1$ dimension. The relaxation delay was kept at 1.2 s for all experiments. The NMR spectroscopic data were processed on a SGI O2 workstation with FELIX 98 software (Byosym, San Diego, CA).

NMR assignment for the quadruplex formed from **C1**: (t5 residue is considered at the 3′ end of the strand) $^1$H NMR ($D_2O$, $T$ = 300 K): δ = 7.54 (T1, H6), 8.19 (G2, H8), 7.87 (G3, H8), 7.80 (G4, H8), 7.20 (t5, H6), 6.02 (T1, H1′), 6.16 (G2, H1′), 6.12 (G3, H1′), 6.09 (G4, H1′), 1.52 (T1, Me), 1.57 (t5, Me), 11.72 (G2, NH), 11.40 (G3, NH), 11.04 (G4, NH) ppm.

NMR assignment for the quadruplex formed from **C3**: (t1 residue is considered at the 5′ end of the strand) $^1$H NMR ($D_2O$, $T$ = 300 K): δ = 7.11 (t1, H6), 8.15 (G2, H8), 7.85 (G3, H8), 7.70 (G4, H8), 7.35 (T5, H6), 6.06 (t1, H1′), 6.11 (G2, H1′), 6.26 (G3, H1′), 6.08 (G4, H1′), 1.62 (t1, Me), 1.60 (T5, Me), 11.81 (G2, NH), 11.39 (G3, NH), 11.18 (G4, NH) ppm.

**UV Thermal Denaturation Experiments:** The concentrations of the synthesized chimeras were determined spectrophotometrically at λ = 260 nm at 80 °C, by use of the molar extinction coefficient calculated for unstacked oligonucleotides [11700 (G); 8800 (T) $cm^{-1}$ $M^{-1}$]. A 10 mM $KH_2PO_4$, 70 mM KCl, 0.2 mM EDTA, pH = 7.0 aq. solution was used for the melting experiments. Melting curves were recorded with a concentration of approximately 1 μ M of single strand in 1 mL of the tested solution in Teflon-sealed quartz cuvettes of 1 cm optical path length. The resulting solutions were then heated at 80 °C for 15 min, then slowly cooled and kept

at 20 °C for 20 min. After thermal equilibration at 20 °C, the UV absorption at λ = 295 was monitored as a function of the temperature, increasing at a rate of 0.5 °C/min. The melting temperatures were determined as the maxima of the first derivatives of the absorbance vs. temperature plots.

**Circular Dichroism:** CD spectra of **C1** and **C3** were recorded in the same buffer as used for UV melting experiments at 5 °C in 0.1-cm pathlength cuvettes. The wavelength was varied from 200 to 340 nm at 5 nm·min$^{-1}$, and the spectra were recorded with a response of 16 s, at 2.0 nm bandwidth and normalised by subtraction of the background scan with buffer. The temperature was kept constant at 5 °C with a thermoelectrically controlled cell holder (JASCO PTC-348).

**Molecular Modelling:** In order to obtain the models of the PNA-containing quadruplexes, the corresponding DNA quadruplex $[d(TGGGT)]_4$ was generated by substitution of the A-tetrad with a T-tetrad in the structure of $[d(AGGGT)]_4$ reported by P. K. Patel et al.[24] All the PNA-containing quadruplexes were then generated by substitution of one or two nucleotidic units with the corresponding PNA monomer into the $[d(TGGGT)]_4$ structure. In particular, the 5′-phosphate groups (in **C3** and **C4** quadruplexes) and 3′-phosphate groups (**C1** and **C2** quadruplexes) of the oligonucleotides were linked to a $NH_2$ group of PNA through a phosphoramidate bond. For all the strands the C-terminus of PNA was capped with a glycine residue. The resulting coordinates of the quadruplexes were then energy-minimised in a vacuum for 200 steps of the steepest descent method, the bases being kept fixed in positions, allowing only the new backbone to relax.

New parameters for the phosphoramidate bond and for the PNA units were added to the Amber force field.[31] The stretch, bend and torsion force constants were assigned to the N-P group by analogy with phosphate constants in the Amber force field. The bond-stretch force constants for NQ-P and CT-NQ (NQ is atom type of nitrogen in phosphoramidate bond) were set slightly higher than in the unmodified backbone in order to model the possible conjugation in the N-P group, by utilisation of the parameters reported by Ding et al.[32] In order to describe the PNA part of the each strand the parameters and atomic charges reported by Shields et al.[33] were used.

The four quadruplexes were neutralised by addition of potassium counter-ions. Each $K^+$ counter-ion was placed on the phosphate bisector 3.5 Å from the phosphorus atom. For PNA units no counter-ions were needed. Each of the quadruplexes with counter-ions was individually placed in a 35Å × 35Å × 35Å box of Monte Carlo TIP3P water,[34] with periodic boundary conditions. The water molecules nearer than 2.8 Å to any solute atom were removed.

The same energy minimisation was used for all the quadruplexes. Each solvated system was energy-minimized for 400 steepest descent steps, the solute being kept fixed in position to allow only the counter-ions and water molecules to reorient themselves favourably around the solute. The solute was then energy-minimised for 400 steepest descent steps under fixed solvent and counter-ion coordinates to remove any local distortion or bad contacts. Finally, solute and solvent molecules were energy-minimised together by use of 1500 steps of the steepest descent method followed by the conjugate method until convergence to a r.m.s. gradient of 0.1 kcal mol$^{-1}$ Å$^{-1}$.

## Acknowledgments

This work is supported by the Italian M.U.R.S.T. (P.R.I.N. 2001) and the Regione Campania (L.41). The authors are grateful to the "Centro Ricerche Interdipartimentale di Analisi Strumentale", C.R.I.A.S. for supplying NMR facilities.

[1] M. Egholm, O. Buchardt, L. Christensen, C. Behrens, S. M. Freier, D. A. Driver, R. H. Berg, S. K. Kim, B. Norden, P. E. Nielsen, *Nature* **1993**, *365*, 566−568.
[2] P. Wittung, P. E. Nielsen, O. Buchardt, M. Egholm, B. Norden, *Nature* **1994**, *368*, 561−563.
[3] E. Uhlmann, A. Peyman, G. Breipohl, D. W. Will, *Angew. Chem. Int. Ed.* **1998**, *37*, 2796−2823, and references cited therein.
[4] H. Knudsen, P. E. Nielsen, *Nucleic Acids Res.* **1996**, *24*, 494−500.
[5] P. Wittung, P. Nielsen, B. Nordén, *Biochemistry* **1997**, *36*, 7973−7979.
[6] P. Wittung, P. Nielsen, B. Nordén, *J. Am. Chem. Soc.* **1999**, *119*, 3189−3190.
[7] J. C. Hanvey, N. J. Peffer, J. E. Bisi, S. A. Thomson, R. Cadilla, J. A. Josey, D. J. Ricca, C. F. Hassman, M. A. Bonham, K. G. Au, S. G. Carter, D. A. Bruckenstein, A. L. Boyd, S. A. Noble, L. E. Babiss, *Science* **1992**, *258*, 1481−1485.
[8] M. A. Bonham, S. Brown, A. L. Boyd, P. H. Brown, D. A. Bruckenstein, J. C. Hanvey, S. A. Thomson, A. Pipe, F. Hassman, J. E. Bisi, B. C. Froehler, M. D. Matteucci, R. W. Wagner, S. A. Noble, L. E. Babiss, *Nucleic Acids Res.* **1995**, *23*, 1197−1203.
[9] B. Datta, C. Schmitt, B. A. Armitage, *J. Am. Chem. Soc.,* in press.
[10] R. G. Kuimelis, A. C. van der Laan, R. Vinayak, *Tetrahedron Lett.* **1999**, *40*, 7671−7674, and references cited therein.
[11] A. C. van der Laan, P. Havenaar, R. S. Oosting, E. Kuyl-Yeheskiely, E. Uhlmann, J. H. van Boom, *Bioorg. Med. Chem. Lett.* **1998**, *8*, 663−668.
[12] M. Borgatti, L. Breda, R. Cortesi, C. Nastruzzi, A. Romanelli, M. Saviano, N. Bianchi, C. Mischiati, C. Pedone, R. Gambari, *Biochem. Pharmacol.* **2002**, *64*, 609−616.
[13] D. A. Barawkar, Y. Kwok, T. W. Bruice, T. C. Bruice, *J. Am. Chem. Soc.* **2000**, *122*, 5244−5250.
[14] E. Ferrer, A. Shevchenko, R. Eritja, *Bioorg. Med. Chem.* **2000**, *8*, 291−297.
[15] R. H. Shafer, I. Smirnov, *Biopolymers* **2001**, *56*, 209−227.
[16] R. Jin, B. L. Gaffney, C. Wang, R. A. Jones, K. J. Breslauer, *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 8832−8836.
[17] D. Capasso, L. De Napoli, G. Di Fabio, A. Messere, D. Montesarchio, C. Pedone, G. Piccialli, M. Saviano, *Tetrahedron* **2001**, *57*, 9481−9846.
[18] J. Feigon, K. M. Koshlap, F. W. Smith, In *Methods in Enzymology* (Ed.: T. L. James), Academic Press, San Diego, **1995**, pp. 225−255.
[19] J. Feigon, in *Encyclopedia of Nuclear Magnetic Resonance* (Ed.: D. M. Grant), John Wiley & Sons Ltd., West Sussex, England, **1996**, pp. 1726−1731.
[20] K. Y. Wang, S. Mc Curdy, R. G. Shea, S. Swaminathan, P. H. Bolton, *Biochemistry* **1993**, *32*, 1899−1904.
[21] J. Feigon, F. W. Smith, *Nature* **1992**, *356*, 164−168.
[22] P. K. Patel, N. S. Bhavesh, R. V. Hosur, *Biochem. Biophys. Res. Commun.* **2000**, *270*, 967−971.
[23] P. K. Patel, R. V. Hosur, *Nucleic Acids Res.* **1999**, *27*, 2457−2464.
[24] P. K. Patel, A. S. R. Koti, R. V. Hosur, *Nucleic Acids Res.* **1999**, *27*, 3836−3843.
[25] J.-L. Mergny, A.-T. Phan, L. Lacroix, *FEBS Lett.* **1998**, *435*, 74−78.
[26] F. Bergmann, W. Bannwarth, S. Tam, *Tetrahedron Lett.* **1995**, *36*, 6823−6826.

[27] M. Piotto, V. Saudek, V. J. Sklenar, *J. Biomol. NMR* **1992**, *2*, 661−665.
[28] J. Jeener, B. H. Meier, P. Bachmann, R. R. Ernst, *J. Chem. Phys.* **1979**, *71*, 4546−4553.
[29] L. Braunschweiler, R. R. Ernst, *J. Magn. Reson.* **1983**, *53*, 521−528.
[30] D. Marion, K. Wuthrich, *Biochem. Biophys. Res. Commun.* **1983**, *113*, 967−874.
[31] W. D. Cornell, P. Cieplak, C. I. Bayly, I. R. Gould, K. M. Merz, D. M. Ferguson, D. C. Spellmeyer, T. Fox, J. W. Caldwell, P. A. Kollman, *J. Am. Chem. Soc.* **1995**, *117*, 5179−5197.
[32] D. Ding, S. M. Gryaznov, D. W. Wilson, *Biochemistry* **1998**, *37*, 12082−12093.
[33] G. C. Shields, C. A. Laughton, M. Orozco, *J. Am. Chem. Soc.* **1998**, *120*, 5895−5904.
[34] W. L. Jorgensen, J. Chandrasekhar, J. D. Madura, R. W. Impey, M. L. Klein, *J. Chem. Phys.* **1983**, *79*, 926−935.

Received April 2, 2003