Pergamon

Tetrahedron 57 (2001) 9481–9486

TETRAHEDRON

Solid phase synthesis of DNA-3′-PNA chimeras by using Bhoc/Fmoc PNA monomers

Domenica Capasso,[a] Lorenzo De Napoli,[b] Giovanni Di Fabio,[b] Anna Messere,[c] Daniela Montesarchio,[b] Carlo Pedone,[d] Gennaro Piccialli[e,*] and Michele Saviano[d]

[a]*Istituto di Microbiologia, Seconda Università di Napoli, S. Aniello a Caponapoli, 80138 Napoli, Italy*
[b]*Dipartimento di Chimica Organica e Biochimica, Università degli Studi di Napoli Federico II, via Cynthia 4, 80126 Napoli, Italy*
[c]*Dipartimento di Scienze Ambientali, Seconda Università di Napoli, via Vivaldi 43, 81100 Caserta, Italy*
[d]*Centro di Studio di Biocristallografia del CNR, via Mezzocannone 6, 80134 Napoli, Italy*
[e]*Dipartimento di Chimica delle Sostanze Naturali, Università degli Studi di Napoli 'Frederico II', Via D. Montesano 49, I-80131 Napoli, Italy*

Received 26 June 2001; revised 3 September 2001; accepted 20 September 2001

Abstract—Oligonucleotides carrying a peptide nucleic acid (PNA) tail at the 3′-end have been efficiently prepared by an on-line automated synthetic protocol exploiting commercially available Bhoc/Fmoc PNA monomers for the assembly of the PNA tract, followed by a deprotection/reprotection of the base protecting groups. The syntheses of the ODN domain in the chimeras have then been performed by standard methods. The hybridization properties of the synthesized chimeras with complementary DNA fragments have been investigated by thermal denaturation experiments.

1. Introduction

Peptide nucleic acids (PNAs) are DNA mimics in which the deoxyribose-phosphate backbone has been replaced by *N*-(2-aminoethyl)glycine units.[1] They can selectively bind to complementary DNA and RNA by Watson–Crick base pairing[2] and are resistant to enzymatic degradation.[3] For these reasons they are promising candidates for the development of gene therapeutic drugs in antisense and/or antigene approaches.[4] However these potential biochemical applications are limited by poor water solubility and inability to activate RNase H in PNA–RNA heteroduplexes.[5] These drawbacks could be overcome by using PNA–DNA hybrids (chimeras) which possess unaltered binding affinity towards complementary nucleic acids and are highly water-soluble and resistant to the degradation by exonucleases.[1b,6]

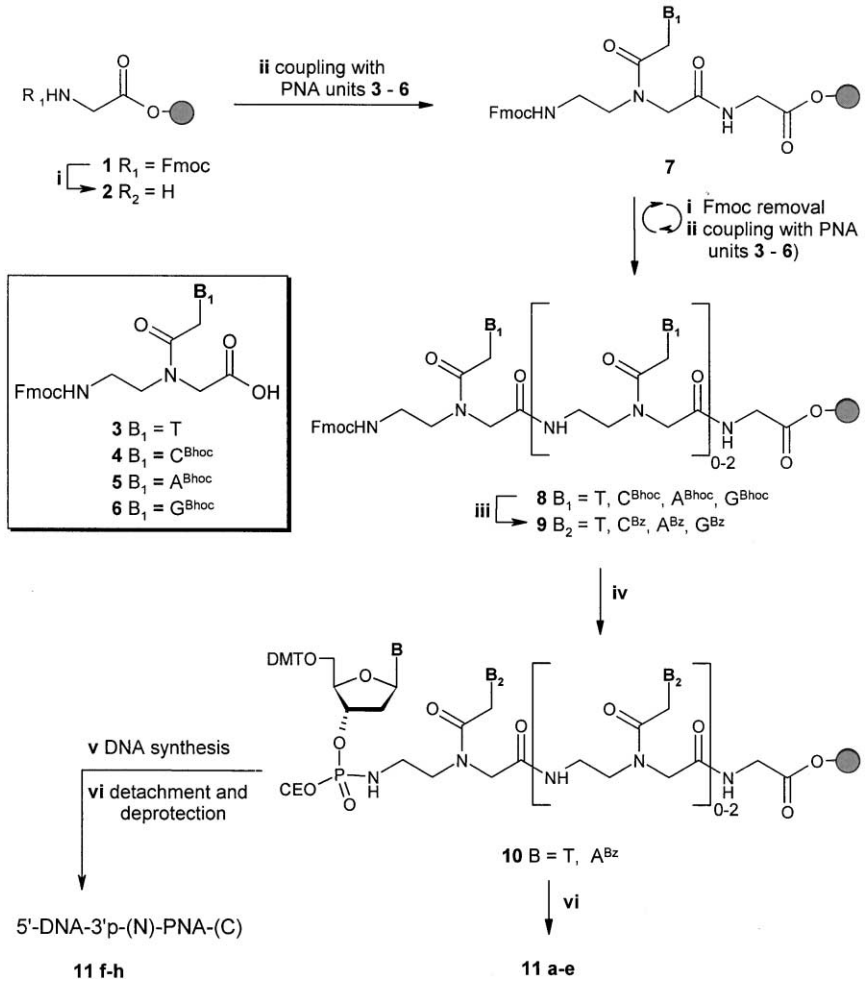
A number of papers have recently appeared in the literature dealing with the synthesis of these chimeras; studies on their hybridization properties[1b,7] and behavior as enzymatic substrates[7c,d] have been also carried out. The type of the linkage at the DNA–PNA junction and the orientation of the PNA tract (N–C vs. C–N) in the chimera seem to be crucial for the effectiveness of the recognition processes.[7a,8]

The synthetic approaches so far proposed for (C)-PNA-(N)-3′-*p*-DNA-5′ are based on chemical template-directed ligation[7a] or 'on line' solid phase strategies.[7c,8,9] In the first approach, the PNA and DNA fragments to be connected are mixed in the presence of complementary DNA or PNA strands as templates and suitable junction promoters (imidazole/EDC) to give the desired chimera. As an alternative to the above solution strategy, many on line solid phase protocols have been described, in which the polymer-supported PNA tract is assembled before the oligodeoxynucleotide (ODN) chain through classical peptide chemistry. Then a stable and structurally favorable junction with the first nucleotide unit is obtained via 3′-phosphoramidate bond formation, followed by standard phosphoramidite synthesis of the DNA tract. The latter pathways require the usage of ad hoc designed, commercially unavailable PNA monomer building blocks carrying a protecting group for the base amino functions which can be removed in alkaline conditions, and an acidic labile group (MMT or DMT) for the transient protection of the 2-ethylamino function. Thus it is possible to follow the same deprotection procedures used in ODN synthesis: acidic treatment by dichloroacetic acid (DCA) addition in the elongation steps and basic conditions (by conc. ammonia treatment), in the final step for detachment from the support and complete deprotection of the oligomer.

In an effort to develop a simpler and more versatile approach to DNA–PNA chimeras, we focused our attention on the possibility of using commercially available PNA

Keywords: nucleic acid analogues; peptide analogues; protecting groups; solid-phase synthesis.
* Corresponding author. Tel.: +39-081-674127/6; fax: +39-081-674393; e-mail: picciall@cds.unina.it

0040–4020/01/$ - see front matter
PII: S0040-4020(01)00944-9



Scheme 1. *Reagents and conditions*: (i) piperidine/DMF (1:4, v/v, 3×10 min, rt); (ii) (a) **3–6** (8 equiv.), HATU (8 equiv.), DIPEA (12 equiv.) in DMF, 1 h, rt, (b) Ac_2O/pyridine (2:3, v/v, 1 h, rt); (iii) (a) 75% TFA in DCM (w/w), 1 h, rt, (b) Ac_2O/pyridine (2:3, v/v, 3 h, rt) or BzCl/pyridine (3:7, v/v, 6 h, rt); (iv) (a) as (i), (b) complete coupling cycle with nucleoside-phosphoramidite (2×20 min, rt); (v) DNA chain assembly on the automated synthesizer; (vi) conc. NH_4OH, 16 or 20 h, 55°C. CE=2-cyanoethyl.

monomers **3–6**, routinely employed for PNA oligomer assembly, as building blocks in an automated synthetic process combining standard phosphoramidite and solid phase peptide chemistry. In such monomers the Fmoc group has been introduced to protect the 2-ethylamino moiety in the growing PNA backbone, while the benzhydryloxycarbonyl (Bhoc) group was chosen as the protecting group for the exocyclic amino functions of A, G and C bases.

2. Results and discussion

In order to render compatible the usage of Bhoc/Fmoc protected PNA monomers with an on-line solid phase synthesis of DNA–PNA chimeras, two conditions have to be fulfilled: (i) the Bhoc protecting group has to be stable during the assembly of the DNA tract by standard phosphoramidite protocols; (ii) the final removal of Bhoc has to be performed under conditions not affecting the ODN domain of the chimera. As far as the first point is concerned, we tested the stability of PNA monomers **3–6** to DCA treatment (2% in DCM), usually required for the removal of the transient DMT protecting group in standard automated DNA assembly. In all cases, TLC analysis, carried out in comparison with authentic samples, showed the Bhoc groups to be completely lost within 25–30 min. Further experiments performed on polymer-supported PNA **7** showed that, under treatment with deblock DCA solution, 5–10% of base deprotection occurred after 3 min (by spectroscopic measurements), which is a not negligible amount considering this step is repeated *n* times in the assembly of an *n*-mer DNA. These results clearly indicated the Bhoc as an unsuitable protecting group for the purines or pyrimidines of PNA monomers in the synthesis of DNA–PNA chimeras in which the PNA tract is assembled, by an on line procedure, before the DNA tract.

To overcome this problem, we have devised an efficient, alternative procedure in which, after the synthesis of the PNA tract, the Bhoc groups were replaced by base-labile protecting groups (acetyl or benzoyl), through a solid phase deprotection–reprotection approach. Then the DNA tract could be synthesized by standard phosphoramidite procedure and a single treatment with conc. aq. ammonia assured the complete, final deprotection of the assembled chimera. In the here proposed synthetic strategy (Scheme 1), Tentagel-OH resin was employed as the solid support and

Table 1. Synthesized chimeras **11**

Chimeras **11**	5′-DNA-3′-PNA	MS data found, (calcd, MH^+)	T_m (ΔT_m), (°C)
a	A-c	641.04 (640.53)	
b	T-g	672.03 (671.54)	
c	T-a	656.10 (655.54)	
d	A-aaa	1215.46 (1215.09)	
e	A-ttt	1188.23 (1188.05)	
f	TGGGGACTTTCCA-ttt	4894.69 (4894.41)	50.0 (+2.0)
g	TGGGGACTTTCCA-aaa	4922.14 (4921.45)	49.0 (+1.4)
h	TCTCTCTCTCTCT-ctc	4704.89 (4704.39)	47.0 (−2.0)

$\Delta T_m = T_m$ duplex with chimera $- T_m$ natural duplex. Capital letters have been used for the DNA bases; small letters indicate the PNA bases.

functionalized with an *N*-Fmoc glycine residue attached through an ester linkage, thus obtaining **1** (0.25 mequiv./g). After deprotection of the amino group of **1**, PNA monomers **3**–**6** were incorporated in solid matrix **2** in the presence of HATU as carboxyl activator giving, after capping of unreacted amino functions, polymer-supported PNA **7**. The coupling cycles were repeated until the PNA sequence of the chimera was completed, giving support **8**. A typical coupling cycle consisted of a single treatment of the NH_2-free support with the chosen PNA monomer and HATU (8 equiv.) for 1 h at rt. The monomers were preactivated by addition of the HATU solution in the presence of DIPEA (12 equiv.) and, after 2 min, this solution was added to the resin. Coupling yields, determined by quantitative monitoring (300 nm) of the fluorene derivative formed by piperidine treatment on weighed amount of the Fmoc-resin, were always in the 92–96% range. Support **8** was then subjected to change of the base protecting group which consisted in treatment with TFA (75%) in DCM, followed, after exhaustive washings, by reaction with acetic anhydride (or benzoyl chloride) in pyridine. Particularly, for PNA tracts not containing G bases, an acetic anhydride/pyridine solution (2:3, v/v, 3 h) was used as the acylating reagent, while in the presence of G bases, benzoyl chloride in pyridine (3:7, v/v, 6 h) was adopted.

Among the various acyl groups which are described in the literature for the protection of the amino groups of heterocyclic bases, the acetyl and the benzoyl groups were tested. It is well documented that the acetyl group can be successfully used for adenine, cytosine and guanine bases protection during the oligonucleotide chain assembly,[10] being removed in alkaline media, even in milder conditions with respect to aromatic acyl groups.[11] Its introduction by treatment with acetic anhydride on the base amino groups is relatively fast for A and C but requires far longer reaction times for G. Therefore in the case of sequences containing G bases in the PNA domain, the more reactive benzoyl chloride was used, assuring the complete N^2-, N^6- and N^4-benzoylation of G, A and C bases, respectively, in 6 h at rt. In this case, prolonged reaction times with aq. ammonia (20 h, 55°C) are required to achieve complete debenzoylation of G bases in the final detachment and deprotection procedure. Quantitation of the Fmoc group on weighed amounts of the resulting supports **9** indicated that such a procedure did not induce cleavage in the PNA chain. On the other hand the analyses (^{1}H NMR and MS data) of the detached PNA materials confirmed the structures of the products; particularly, in the NMR spectra, all the signals of the PNA tract and of the glycine tail were observed in the expected ratio. Once assembled the PNA tract of the chimera, the terminal amino function was deprotected and then coupled with the first nucleotide building block to allow the synthesis of the DNA domain. The DNA chain assembly was performed on an automated DNA synthesizer using standard phosphoramidite chemistry at 10 μmol scale. DMT quantitation confirmed acceptable yields (90–94%) in the first coupling step, forming the phosphoramidate DNA-3′-PNA junction, and standard yields (98–99%) in the growing of the DNA tract, leading to supported chimera **10**. It is to be noted that in the coupling with the first phosphoramidite unit longer reaction times (30 min) and two coupling cycles were used. To test the feasibility of the proposed synthetic pathway, a number of small hybrids (**11a**–**e**, Table 1) were synthesized, purified by reverse-phase HPLC and their structures confirmed by ^{1}H NMR and MS data. Following this synthetic route, we then prepared three 16-base long chimeras having three PNA monomers at the 3′-end (**11f**–**h**). The crude detached conjugates were purified by HPLC on an anion exchange Nucleogen column (Fig. 1) and desalted by gel filtration chromatography on a Sephadex G25 column eluted with H_2O. The isolated products were checked for purity by HPLC on a RP18 column, showing the compounds to be more than 98% pure. MALDI-TOF mass spectrometry was employed to characterize the synthesized chimeras.

Thermal denaturation experiments have been carried out to investigate the hybridization properties of the synthesized DNA-3′-PNA chimeras with complementary DNA fragments. As evaluated by comparison of the melting temperatures relative to the duplexes formed by chimeras **11f**–**h** and the respective complementary DNA fragments with those of the duplexes formed by the corresponding all DNA sequences (see Table 1), we found that the affinity of the chimeras towards target DNA were quite affected by the presence of the PNA structure, exhibiting ΔT_m from −2 to +2°C. In relation with the corresponding all DNA sequences, these data do not appear to be easily explainable if not in terms of sequence effects, probably inducing characteristic differences in the conformation at the DNA/PNA junction, critical for the chimeras to show favorable binding properties. Further studies are currently in progress in our laboratories to extend the repertoire of available DNA/PNA sequences and get a deeper insight in the behavior of such chimeras, in view of their possible use in in vitro and in vivo experiments as potential therapeutic agents.

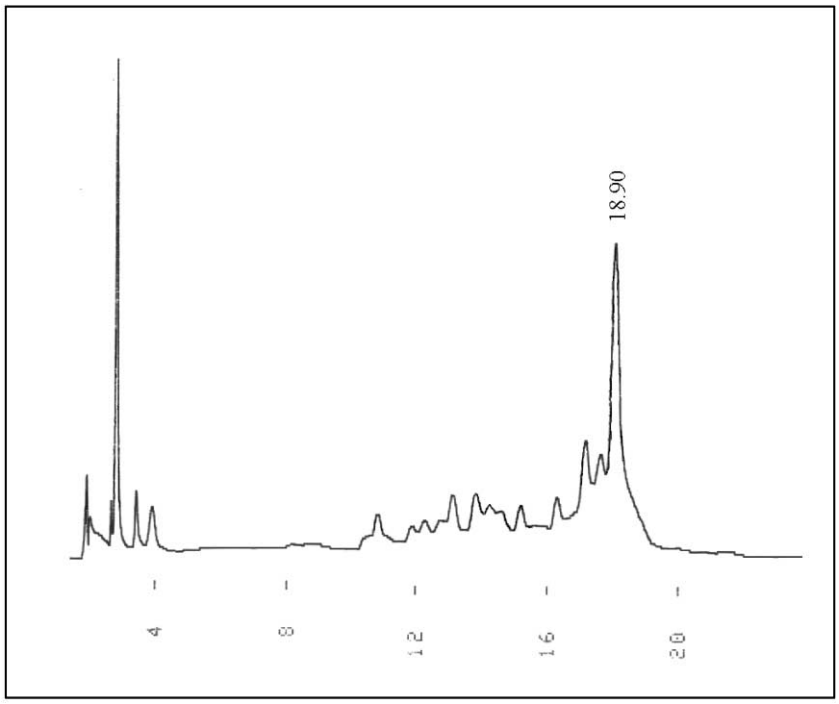


Figure 1. HPLC profile of crude **11g**, purified on a Nucleogel SAX column; buffer A: 20 mM KH_2PO_4 aq. solution, pH=7.0, containing 20% (v/v) CH_3CN; buffer B: 1 M KCl, 20 mM KH_2PO_4 aq. solution, pH=7.0, containing 20% (v/v) CH_3CN; elution: linear gradient from 0 to 100% B in 30 min; flow rate 1.0 mL/min.

3. Conclusions

An efficient on-line automated solid phase synthesis of (C)-PNA-(N)-3′-*p*-DNA-5′ chimeras has been here described. Several small hybrids have been synthesized and fully characterized to test the feasibility of the proposed synthetic protocol, based on the usage of commercially available Bhoc/Fmoc PNA monomers in the assembly of the PNA tract of the chimera. Since the Bhoc protecting group was not completely stable to the DCA treatments, required by the DNA chain assembly procedure, a simple and convenient deprotection/reprotection approach has been developed, involving the removal of such protecting groups on the assembled PNA tract, followed by peracetylation or perbenzoylation of the exocyclic amino functions of the bases. Standard phosphoramidite chemistry could therefore be exploited in the growing of the DNA domain. Three 16 bases DNA/PNA chimeras were then synthesized, characterized by MALDI TOF experiments and tested in duplex formation experiments with complementary DNA oligomers.

4. Experimental

4.1. Materials and methods

NMR spectra were recorded on Bruker WM-400 and Varian Gemini 300 spectrometers. All chemical shifts are expressed in ppm with respect to the residual solvent signal. The solid support functionalizations and PNA tract assemblies were carried out in a short glass column (5 cm length, 1 cm i.d.) equipped with a sintered glass filter, a stopcock and a cap. The oligonucleotides were assembled on a Millipore Cyclone Plus DNA synthesizer, using commercially available 3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite 2′-deoxyribonucleosides as building blocks.

The following abbreviations were used throughout the text: benzhydryloxycarbonyl (Bhoc), fluorenylmethoxycarbonyl (Fmoc), 4-monomethoxytrityl (MMT), 4,4′-dimethoxytrityl (DMT), dichloroacetic acid (DCA), dichloromethane (DCM), *O*-7-azabenzotriazol-1yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU), diisopropyethylamine (DIPEA), trifluoroacetic acid (TFA), *N*,*N*-dimethylformamide (DMF), *N*,*N*-dicyclohexylcarbodiimide (DCCI), *N*-hydroxybenzotriazole, (HOBt), benzoyl chloride (BzCl).

HPLC analyses and purifications were performed on a Beckman System Gold instrument equipped with a UV detector module 166 and a Shimadzu Chromatopac C-R6A integrator. Thermal denaturation experiments were carried out on a Jasco V-530 UV spectrophotometer with detection at λ=260 nm and equipped with a Jasco ETC-505T temperature controller unit. Tentagel-OH resin was purchased from Rapp Polymere, Tubingen, Germany.

4.2. Functionalization of the resin. Supports 1 and 2

Fmoc-Gly-OH (400 mg, 1.35 mmol) and DCCI (278 mg, 1.35 mmol) were dissolved in DMF (3 mL). After 10 min at rt the resulting mixture and 206 mg of HOBt (1.35 mmol) were added to 500 mg of Tentagel resin (0.135 mmol of hydroxy groups), previously washed with DMF. The mixture was kept at rt for 16 h under shaking. The support was filtered and washed with DMF and Et_2O and then dried under reduced pressure, thus obtaining **1** (0.25 mequiv./g).

The incorporation of the glycine residue was calculated by quantitative UV measurement (300 nm) of the fluorene derivative released by piperidine/DMF treatment on *1* (1:4, v/v, 3×10 min) and leading to support **2**.

4.3. Coupling with PNA monomers (general procedure). Supports 7 and 8

Support **2** (100 mg, 0.025 mmol) was washed with DMF and then left in contact with a DMF solution (2 mL) of the appropriate PNA monomer (**3**–**6**, 0.20 mmol) in the presence of 76 mg (0.20 mmol) of HATU and 51 μL (0.30 mmol) of DIPEA for 1 h at rt under shaking. After washings with DMF on support **7**, a capping procedure by addition of acetic anhydride in pyridine (2:3, v/v, 1 h at rt) was carried out. Fmoc removal was performed as reported before by piperidine treatment. Particularly for A and T PNA monomers, a DMF/CH_3CN (1:1, v/v) solution was used. The same deprotection and coupling procedures were used in the elongation of the PNA tract, thus furnishing type-**8** support. The coupling yields, measured as for support **2**, resulted to be 92–96%. On supports **7** the loss of the Bhoc group, induced by DCA solution treatments (3 min), was calculated by spectroscopic analysis. The DCA eluate (2% in DCM), after drying, was dissolved in conc. H_2SO_4 and the contained Bhoc cation, quantified at 442 nm, resulted in the 5–10% range. A further DCA treatment (3 min) induced deprotection of the bases almost to the same extent.

4.4. Base deprotection–reprotection procedure. Support 9

Support **8** (50 mg, 0.01 mmol) was washed with DCM and then treated with a solution (1 mL) of TFA (75%) in DCM for 1 h at rt. After exhaustive washings with DCM and pyridine, 1 mL of a solution of Ac_2O in pyridine (2:3, v/v) was added to the support not containing G bases and the mixture shaken for 3 h at rt. For G-containing supports benzoyl chloride in pyridine (1 mL, 3:7, v/v) as the acylating mixture and longer reaction times (6 h, rt) were employed. The resin was then washed with pyridine, DMF and Et_2O and dried under reduced pressure, thus furnishing support **9**.

4.4.1. Synthesis of products 11a–e: general procedure. Support **9** (50 mg, 0.01 mmol), transferred in the standard short column used in the DNA synthesizer, was washed with DMF and then left in contact with 20% piperidine solution in DMF (3×10 min, rt) by using a glass syringe. After washings with DMF, the column containing the support was placed into the DNA synthesizer and exhaustively washed with CH_3CN. The coupling step with the first 5′-*O*-DMT-3′-*O*-(2-cyanoethyl)phosphoramidite nucleoside building block was performed following the standard phosphoramidite chemistry. This coupling step, which led to support **10** (90–94% yield), was carried out using a solution of the amidite unit (40 mg/mL) in CH_3CN using longer reaction times (20 min) and was repeated twice. After washings with CH_3CN and final DMT removal, the support was treated with conc. aq. ammonia for 16 h (or 20 h for G containing sequences) at 55°C. The filtered solution and H_2O/MeOH (1:1, v/v) washings were concentrated under reduced pressure and purified by HPLC on a Nucleosil 100-5 C_{18} column (4.6×250 mm, 7 μm) eluted with a linear gradient of CH_3CN in TEAB buffer (0.1 M, pH 7.0). The final products were lyophilized and characterized by NMR and MS data.

Compound **11a**: ^{1}H NMR (400 MHz, D_2O), δ 8.37 (s, 1H, H-2 adenine); 8.34 (s, 1H, H-8 adenine); 7.49 (d, *J*=7.2 Hz 1H, H-6 cytosine); 6.53 (dd, *J*=7.3 and 7.4 Hz, 1H, H-1′); 6.02 (d, *J*=7.2 Hz, 1H, H-5 cytosine); 5.00 (m, 1H, H-3′); 4.64 (s, 2H, CH_2 Gly); 4.38 (s, 2H, CH_2 PNA); 4.11 (complex signal, 3H, H-4′ and H_2-5′); 4.00 (s, 2H, CH_2 PNA); 3.83 (m, 2H, CH_2 PNA); 3.38 (m, 2H, CH_2 PNA), 2.75 (m, 2H, H_2-2′).

Compound **11b**: ^{1}H NMR (400 MHz, D_2O), δ 7.91 (s, 1H, H-2 guanine); 7.69 (s, 1H, H-6 thymine); 6.22 (dd, *J*=6.4 and 6.5 Hz, 1H, H-1′); 4.96 (m, 1H, H-3′); the signal relative to glycine CH_2 is submerged by the solvent signal; 4.54 (s, 2H, CH_2 PNA); 4.05 (complex signal, 3H, H-4′ and H_2-5′); 3.80 (s, 2H, CH_2 PNA); 3.62 (m, 2H, CH_2 PNA); 3.20 (m, 2H, CH_2 PNA); 2.85 (m, 2H, H_2-2′); 1.88 (s, 3H, CH_3 thymine).

Compound **11c**: ^{1}H NMR (400 MHz, D_2O), δ 8.41 (s, 1H, H-2 adenine); 8.20 (s, 1H, H-8 adenine); 7.58 (s, 1H, H-6 thymine); 6.26 (dd, *J*=6.5 and 6.5 Hz, 1H, H-1′); 5.03 (m, 1H, H-3′); the signal relative to glycine CH_2 is submerged by the solvent signal; 4.62 (s, 2H, CH_2 PNA); 4.08 (complex signal, 3H, H-4′ and H_2-5′); 3.96 (s, 2H, CH_2 PNA); 3.64 (m, 2H, CH_2 PNA); 3.21 (m, 2H, CH_2 PNA); 2.50 (m, 2H, H_2-2′); 1.93 (s, 3H, CH_3 thymine).

Compound **11d**: ^{1}H NMR (400 MHz, CD_3OD), δ 8.53, 8.53, 8.51, 8.33 (s's, 1H each, H-2 adenines); 8.19, 8.14, 8.14, 8.12 (s's, 1H each, H-8 adenines); 6.47 (dd, *J*=7.0 and 6.9 Hz, 1H, H-1′); 5.04 (m, 1H, H-3′); 4.64 (bs, CH_2 Gly); 4.31–4.03 (complex signals, 9H, CH_2 PNAs, H-4′, and H_2-5′); 3.96 (m, 6H, CH_2 PNAs); 3.81 (m, 6H, CH_2 PNAs); 3.40–3.50 (m, 6H, CH_2 PNAs); 2.80 and 2.90 (m's, 1H each, H_2-2′).

Compound **11e**: ^{1}H NMR (400 MHz, CD_3OD), δ 8.37 (s, 1H, H-2 adenine); 8.18 (s, 1H, H-8 adenine); 7.32, 7.29, 7.27 (s's, 1H each, 3H, H-6 thymine); 6.47 (dd, *J*=7.0 and 7.1 Hz, 1H, H-1′); 4.97 (m, 1H, H-3′); the signal relative to glycine CH_2 is submerged by the solvent signal; 4.65–4.50 (complex signals, 6H, CH_2 PNAs); 4.30–4.15 (complex signals, 3H, H-4′ and H_2-5′); 3.85–3.45 (complex signals, 18H, CH_2 PNAs); 2.73 (m, 2H, H_2-2′); 1.98, 1.89, 1.88 (s's, 3H each, CH_3 thymine).

4.4.2. Synthesis of chimeras 11f–h. ODN chain assembly was performed on 50 mg (0.09–0.01 mmol) of support **10** on an automated DNA synthesizer following the standard phosphoramidite procedure with final DMT removal. Three sequences were assembled (**11f**–**h**) observing coupling yields always greater than 98%. Oligomers **11f**–**h** were detached from the support and deprotected by conc. ammonia treatment as described for **11a**–**e**. The supernatant was filtered and the support washed with H_2O and a solution of H_2O/MeOH. The combined filtrates and washings were concentrated in vacuo, redissolved in H_2O and analyzed 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 1.0 mL/min, was used. The isolated oligomers, having the following retention times: **11f**=20.30 min; **11g**=18.90 min; **11h**=19.60 min, were collected and successively desalted by gel filtration on a Sephadex G25 column eluted with H_2O. By HPLC analysis on a Partisphere Whatman RP18 analytical column (125×4.0 mm^2, 5 μm), the isolated oligomers resulted to be more than 98% pure.

4.5. Thermal denaturation experiments

The concentration of the synthesized ODNs was determined spectrophotometrically at λ=260 nm and at 80°C, using the molar extinction coefficient calculated for the unstacked oligonucleotide using the following extinction coefficients: 15400 (A); 11700 (G); 7300 (C); 8800 (T) $cm^{-1}\,M^{-1}$.[12] A 100 mM NaCl, 10 mM NaH_2PO_4, aq solution at pH=7.0 was used for the melting experiments. Melting curves were recorded using a concentration of approximately 1 μM for each strand in 1 mL of the tested solution in Teflon stoppered quartz cuvettes of 1 cm optical path length. The resulting solutions were then allowed to heat at 80°C for 15 min, then slowly cooled and kept at 5°C for 20 min. After thermal equilibration at 10°C, UV absorption at λ=260 nm was monitored as a function of the temperature, increased at a rate of 0.5°C/min, typically in the range 20–80°C. The melting temperatures were determined as the maxima of the first derivative of absorbance vs. temperature plots.

Acknowledgements

We thank MURST (PRIN), CNR and Regione Campania (Legge 41) for grants in support of this investigation and Centro di Metodologie Chimico-Fisiche (CIMCF), Università di Napoli 'Federico II', for the NMR facilities.

References

1. (a) Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O. *Science* **1991**, *254*, 1497–1500. (b) Uhlmann, E.; Peyman, A.; Breipohl, G.; Will, D. W. *Angew. Chem., Int. Ed. Engl.* **1998**, *37*, 2796–2823.
2. Egholm, M.; Buchardt, O.; Christensen, L.; Beherens, C.; Freier, S. M.; Driver, D. A.; Berg, R. H.; Kim, S. K.; Norden, B.; Nielsen, P. E. *Nature* **1993**, *365*, 566–568.
3. (a) Uhlmann, E.; Peyman, A. *Chem. Rev.* **1990**, *90*, 543–584. (b) Hyrup, B.; Nielsen, P. E. *Bioorg. Med. Chem.* **1996**, *4*, 5–23.
4. (a) Knudsen, H.; Nielsen, P. E. *Anti-Cancer Drugs* **1997**, *8*, 113–118. (b) Uhlmann, E.; Peyman, A.; Breipohl, G.; Will, D. W. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 2632–2638.
5. Knudsen, H.; Nielsen, P. E. *Nucleic Acids Res.* **1996**, *24*, 494–500.
6. Uhlmann, E. *Biol. Chem.* **1998**, *379*, 1045–1052.
7. For example see: (a) Koppitz, M.; Nielsen, P. E.; Orgel, L. E. *J. Am. Chem. Soc.* **1998**, *120*, 4563–4569. (b) van der Laan, A. C.; Havenaar, P.; Oosting, R. S.; Kuyl-Yeheskiely, E.; Uhlmann, E.; van Boom, J. H. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 663–668. (c) Verheijen, J. C.; van Roon, A.-M.; van der Laan, A. C.; van der Marel, G. A.; van Boom, J. H. *Nucleosides Nucleotides* **1999**, *18*, 493–508. (d) Kuimelis, R. G.; van der Laan, A. C.; Vinayak, R. *Tetrahedron Lett.* **1999**, *40*, 7671–7674.
8. Greiner, B.; Breipohl, G.; Uhlmann, E. *Helv. Chim. Acta* **1999**, *82*, 2151–2159.
9. van der Laan, A. C.; Brill, R.; Kuimelis, R. G.; Kuyl-Yeheskiely, E.; van Boom, J. H.; Andrus, A.; Vinayak, R. *Tetrahedron Lett.* **1997**, *38*, 2249–2252.
10. Pitsch, S.; Weiss, P. A.; Wu, X.; Ackermann, D.; Honegger, T. *Helv. Chim. Acta* **1999**, *82*, 1753–1761.
11. (a) Koster, H.; Kulikowsky, K.; Liese, T.; Heikens, W.; Kohli, V. *Tetrahedron* **1981**, *37*, 363–369. (b) Schulhof, J. C.; Molko, D.; Teoule, R. *Nucleic Acids Res.* **1987**, *15*, 397–416.
12. (a) *Handbook of Biochemistry and Molecular Biology*, Fasman, G. D., Ed.; 3rd ed., *Nucleic Acids*, CRC: Boca Raton, 1975; Vol. 1, p. 589. (b) Cantor, C. R.; Warshaw, M. M.; Shapiro, H. *Biopolymers* **1970**, *9*, 1059–1077.