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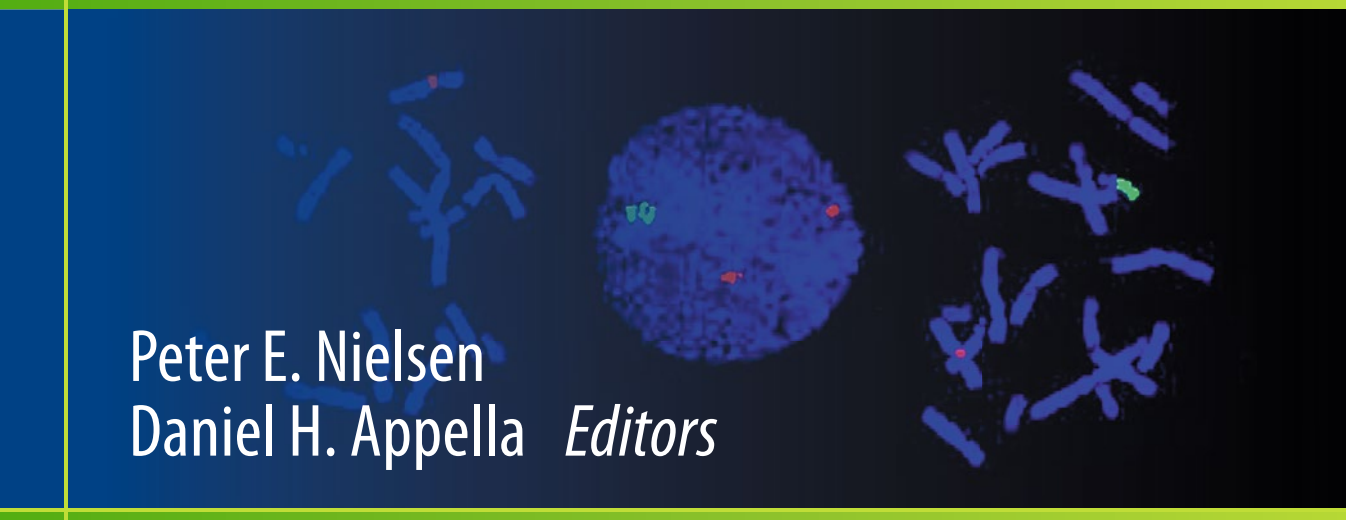

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Daniel H. Appella *Editors*

Peptide Nucleic Acids

Methods and Protocols

Second Edition

Humana Press

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Peptide Nucleic Acids

Methods and Protocols

Second Edition

Edited by

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Preface

Peptide nucleic acids "celebrated" the 20th anniversary in 2011. Since their discovery the chemistry has been both intensively and extensively explored, and many interesting new derivatives and analogs in terms of nucleic acid recognition specificity and affinity have emerged. Also great ingenuity in exploiting the unique properties of PNAs for a wide variety of applications within drug discovery, medical diagnostics, chemical biology, and nanotechnology has unfolded. In the present volume we have attempted to exemplify and illustrate recent exciting advances in PNA chemistry and applications as a complement to the first edition of this book. Therefore, we hope that it shall serve both as a source of useful specific methods and protocols as well as a source of inspiration for future developments.

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Chapter 1

MiniPEG-γPNA

Arunava Manna, Srinivas Rapireddy, Raman Bahal, and Danith H. Ly

Abstract

Peptide nucleic acids (PNAs) are attractive, as compared to other classes of oligonucleotides that have been developed to date, in that they are relatively easy to synthesize and modify, hybridize to DNA and RNA with high affinity and sequence selectivity, and are resistant to enzymatic degradation by proteases and nucleases; however, the downside is that they are only moderately soluble in aqueous solution. Herein we describe the protocols for synthesizing the second-generation γPNAs, both the monomers and oligomers, containing MiniPEG side chain with considerable improvements in water solubility, biocompatibility, and hybridization properties.

Key words Chiral PNA, Backbone modification, Conformational preorganization, Water solubility

1 Introduction

Peptide nucleic acids (PNAs) are attractive, as compared to other classes of oligonucleotides that have been developed to date, in that they are relatively easy to synthesize and modify, hybridize to DNA and RNA with high affinity and sequence selectivity, and are resistant to enzymatic degradation by proteases and nucleases; however, the downside is that they are only moderately soluble in aqueous solution. Herein we describe the protocols for synthesizing the second-generation γPNAs, both the monomers and oligomers, containing MiniPEG side chain with considerable improvements in water solubility, biocompatibility, and hybridization properties.

PNAs hold considerable promise as molecular tools for basic research in biology, biotechnology, and molecular engineering, as well as therapeutic and diagnostic reagents for the treatment and detection of genetic diseases [1–7], because of their strong binding affinity and sequence selectivity, resistance to proteases and nucleases [5], and ease [8, 9] and flexibility of synthesis [10].

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However, the drawback, as compared to other classes of oligonucleotides developed to date, is water solubility [11, 12]. Because of their charge-neutral backbone, the very essence that endows PNAs with many of their appealing attributes, they are only moderately soluble in aqueous solution. While this is not an issue for most in vitro and cell culture work, this inherent property posts a considerable challenge for their handling and processing at elevated concentrations—for instance, in the development of microarrays and surface-bound applications, because of their propensity to aggregate and collapse onto the surface, causing poor and nonspecific binding [13, 14], and in biology and medicine, in part because of the concern for off-target binding and cytotoxicity [12].

Several approaches have so far been undertaken to try to address this issue, including covalent attachment of charged amino acid residues [15–17]; installation of polar groups in the backbone [18], carboxymethylene bridge [19], and nucleobases [20]; replacement of the original pseudopeptide backbone with a negatively charged scaffold [21, 22]; conjugation of large-molecular-weight polyethylene glycol (PEG) to one or both of the termini [14, 23]; fusion of PNA to DNA to generate a chimeric oligomer [24–28]; and complete redesign of the backbone skeleton [29]. While some success has been achieved with these various strategies, it is often at the expense of binding affinity and/or sequence selectivity and, in some cases, requires an elaborate synthetic scheme. Herein we report the protocols for synthesizing diethylene glycol (or MiniPEG)-containing γPNA monomers and oligomers [30], with considerable improvements in water solubility, biocompatibility, and hybridization properties over the original design. Unlike the achiral counterparts, chiral γPNA oligomers [31–35] adopt a preferred conformation, either a right-handed or a left-handed helix, depending on the stereochemistry at the γ-backbone position. Those derived from L-amino acids adopt a right-handed helix and hybridize to DNA and RNA with high affinity and sequence selectivity, while those derived from unnatural D-amino acids do not; however, they do hybridize to their partners with affinity and sequence selectivity and level of orthogonality. These latter properties make them attractive as molecular recognition codes for organizing and programming molecular self-assembly. The solid-phase synthesis protocols described herein differ considerably from those originally reported by Christensen and co-workers [36], in that they have been optimized for high-throughput synthesis, whereby several critical steps, including neutralization and capping, have been omitted in order to expedite the process and reduce the cost of material consumption and also to enable the synthesis of thioester-containing γPNA oligomeres.

2 Material

Further purification of commercial reagents is not necessary. Prepare dry solvents by standard methods. Perform chromatography using standard-grade silica gel and TLC with precoated silica gel plates. Carefully follow all waste disposal regulations when disposing waste solvents and materials.

2.1 Chemicals

1. DCC: *N,N′*-Dicyclohexylcarbodiimide.
2. DCM: Dichloromethane.
3. DCU: *N,N′*-Dicyclohexylurea.
4. DhBtOH: 3-Hydroxy-4-oxo-3, 4-dihydro-1, 2, 3-benzotriazin.
5. DIAD: Diisopropyl azodicarboxylate.
6. DIPEA: Di-isopropylethylamine.
7. DME: Dimethyl ether.
8. DMF: *N,N*-Dimethylformamide.
9. HATU: 2-(1*H*-7-Azabenzotriazol-1-yl)-1, 1, 3, 3-tetramethyl uranium hexafluorophosphate methanaminium.
10. HBTU: *O*-Benzotriazole-*N, N, N′, N′*-tetramethyl-uronium-hexafluoro-phosphate.
11. nMDCHA: *n*-methyl dicyclohexylamine.
12. NMM: *N*-Methylmorpholine.
13. NMP: *N*-Methyl-2-pyrrolidone.
14. TFA: Trifluoroacetic acid.
15. TFMSA: Trifluoromethanesulfonic acid.
16. THF: Tetrahydrofuran.

2.2 Kaiser Solutions

1. K*aiser A*: Mix 980 μL pyridine with 100 μL phenol/EtOH (4:1) and 20 μL KCN stock solution. (*KCN stock solution*: Dissolve 0.65 mg KCN in 10 mL water, then take 2 mL of that solution, and mix it with 98 mL water.)
2. *Kaiser B*: Dissolve 5 g ninhydrin in 100 mL EtOH.
3. Kaiser test: Add one drop of Kaiser A and one drop of Kaiser B to an Eppendorf tube containing a small amount of resin, and heat the mixture at 90 °C for 2 min in a heating block. Blue color indicates the presence of free amines.

2.3 Coupling Solutions (Figs. 1 and 2)

1. Solution A: 0.2 M Boc-Lys-2-Cl-Z-OH in NMP. Dissolve 7.6 mg Boc-Lys-2-Cl-Z-OH in 100 μL NMP.
2. Solution B: 0.500 M DIEA in pyridine. Dissolve 87 μL DIEA in 913 μL pyridine.

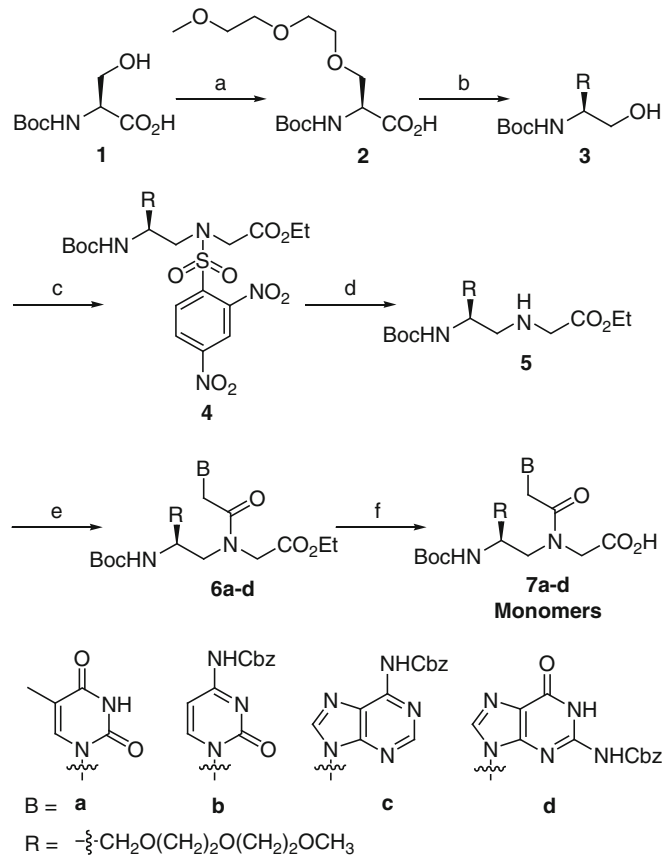

Fig. 1 *Reagents and conditions*: (*a*) NaH, $BrCH_2CH_2OCH_2CH_2OCH_3$, DMF, 0 °C, 68 %; (*b*) isobutyl chloroformate, $NaBH_4$, NMM, DME, 0 °C → rt, 84 %; (*c*) DIAD, 2,4-dinitrobenzenesulfonyl glycine ethyl ester, TPP, THF, 0 °C → rt, 56 %; (*d*) *n*-propylamine, CH_2Cl_2, rt, 81 %; (*e*) carboxymethylene nucleobase, DCC, DhObtOH, DMF, 50 °C, 67–85 %; (*f*) 2 M NaOH/THF (1:1), 0 °C, 85–98 %

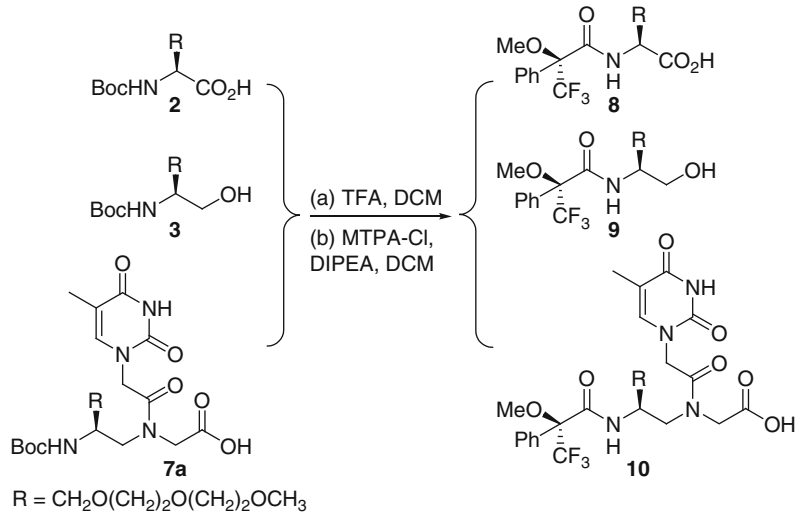

Fig. 2 Monomer coupling

DATE: _____ **PNA SYNTHESIS FORM** **PNA#: _____**

100mg Resin (*Suitable for Preparing Thioester as Well*)

Sequence (C→N)																			
TFA/m																			
TFA/m-cresol (5min)																			
DCM (3X)																			
DMF (3X)																			
Kaiser Test (B)																			
Coupling (15min)																			
DMF (4X)																			
DCM																			
Kaiser Test (Y)																			

Coupling solution : 300μL 0.2M monomer (NMP) + 150 μL 0.52M DIEA (DMF) + 150 μL 0.39M HBTU (DMF), activate for 3 min.

0.39M HBTU (DMF): Dissolve 740mg HBTU in 5mL anhydrous DMF
0.52M DIEA (DMF): Add 362 μLDIEA to 3.638mL anhydrous DMF

0.2M Monomers: Dissolve the following amounts in 300μL NMP

Boc-Lys(2-Cl-Z)-OH: 25mg; Boc-Lys(Fmoc)-OH: 28mg; Boc-mini-PEG: 16mg; Boc-mini-PEG-3: 18mg; 3-TP(Trt): 21mg; Carboxy-TAMRA: 28mg

Boc-PNA-Monomers : A^{Cbz}: 32mg; C^{Cbz}: 30mg; G^{Cbz}: 33mg; T: 23mg
MiniPEG-γ PNA-Monomers: A^{Cbz}:40mg; C^{Cbz}: 38mg; G^{Cbz}: 41mg; T: 31mg

Fig. 3 PNA synthesis form

3. Solution C: 0.202 M HATU in NMP. Dissolve 15.36 mg HATU in 200 μL NMP.
4. Solution D: Mix 45 μL Solution A with 46 μL Solution B and 159 μL NMP.
5. Solution E: Mix 55 μL Solution C with 195 μL NMP.
6. Capping solution: Mix 2 mL NMP with 2 mL pyridine and 1 mL acetic anhydride.
7. Deprotection solution: Mix 95 mL TFA with 5 mL m-cresol.

2.4 Monomer Coupling Solutions (See Note 2*)*

1. 0.2 M Monomer in DMF (**Solution A**): *See* Fig. 3 chart for the exact amount of each monomer.
2. 0.52 M DIEA in DMF (**Solution B**): Mix 362 μL DIEA with 3.638 mL anhydrous DMF.
3. 0.39 M HBTU in DMF (**Solution C**): Dissolve 740 mg HBTU in 5 mL anhydrous DMF.

2.5 Cleavage from Resin

1. Cleavage cocktail: 100 μL m-cresol, 100 μL thioanisole, 200 μL TFMSA, and 600 μL TFA.
2. Deprotection solution: m-cresol/TFA: 5/95.

3 Methods

3.1 Monomer Synthesis

Carry out all moisture-sensitive reactions under nitrogen atmosphere.

3.1.1 Boc-(2-(2-Methoxyethoxy) Ethyl)-L-Serine [2]

1. Dissolve Boc-L-Ser-OH (4.05 g, 19.6 mmol) in anhydrous DMF (30 mL).
2. Use addition funnel, and add the above solution dropwise over the course of 3 h to a suspension of NaH (60 %, 1.7 g, 42.6 mmol) in anhydrous DMF (80 mL) at 0 °C while stirring.
3. Upon completing **step 2**, add 1-bromo-2-(2-methoxyethoxy) ethane (6.4 mL, 42.6 mmol) to the reaction mixture at once at 0 °C. Remove the ice bath, and gradually allow the reaction mixture to warm to room temperature and continue stirring for another 3 h.
4. After confirming that the reaction is complete by TLC, quench the reaction by adding cold water (100 mL) to the mixture. Then evaporate the solvents under reduced pressure (maintaining the water bath temperature below 50 °C at all times, *see* **Note 3**).
5. Add 20 mL of water to the residue in the flask and then acidify with 5 % aqueous HCl to pH ~3.
6. Extract the aqueous layer with ethyl acetate (5 × 100 mL), dry the combined organic layers over anhydrous Na_2SO_4, and then remove the solvent under reduced pressure.
7. Purify the crude residue by column chromatography using EtOH/EtOAc (5/95) solvent mixture as an eluent (R_f: 0.2, TLC). The product is a colorless liquid. The yield should be ~68 % (4.1 g).

3.1.2 Boc-(2-(2-Methoxyethoxy) Ethyl)-L-Serine-ol [3]

1. Add NMM (1.43 mL, 13.0 mmol) to a stirred solution of compound **2** (4 g, 13.0 mmol) in 20 mL of DME at 0 °C, and allow the mixture to stir for another 10 min.
2. Add isobutyl chloroformate (1.76 mL, 13.0 mmol) dropwise to the solution in **step 1**.
3. After stirring for 30 min, white precipitate should form. At this point, filter off the precipitate and wash with DME (2 × 10 mL). *Note that the product is in the flow-through.*
4. Place the filtrate in 250-mL round-bottom flask and chill in an ice bath while stirring.
5. To the solution in **step 4**, slowly add $NaBH_4$ (0.741 g, 19.5 mmol; dissolved in 10 mL water) (*see* **Note 2**), and continue to stir the mixture for 30 min.
6. Extract the reaction mixture with ethyl acetate (3 × 100 mL), wash the combined organic layers with brine, dry over anhydrous

Na_2SO_4, evaporate the solvent under reduced pressure, and then purify the crude mixture by silica gel column chromatography using EtOAc as an eluent (R_f: 0.25, TLC). The product is a colorless liquid. The yield should be ~84 % (3.2 g).

3.1.3 Boc-(2-(2-Methoxyethoxy) Ethyl)-L-Serine-Ψ[CH_2N(o,p-diNBS)] Gly-OEt [4]

1. Sequentially add 2,4-dinitrobenzenesulfonyl glycine ethyl ester (3.53 g, 10.5 mmol, *see* **Note 4**) and triphenyl phosphine (2.73 g, 10.5 mmol) to a stirred solution of compound **3** (3.1 g, 10.5 mmol) in anhydrous THF (20 mL) at 0 °C.
2. Add DIAD (1.48 mL, 10.5 mmol) dropwise over the course of 30 min to the resulting mixture.
3. Allow the reaction mixture to warm to room temperature and then stir overnight.
4. Evaporate the solvent under reduced pressure, and purify the oily residue by column chromatography using EtOAc/hexane (60/40) mixture as an eluent (R_f: 0.40, TLC). The product is a yellow solid. The yield should be ~56 % (3.6 g).

3.1.4 Boc-(2-(2-Methoxyethoxy) Ethyl)-L-Serine-Ψ[CH_2N]Gly-OEt [5]

1. Add *n*-propyl amine (7.0 mL, 85.4 mmol) dropwise to a stirred solution of compound **4** (2.6 g, 4.2 mmol) in DCM (20 mL) at room temperature and continue to stir for another 20 min.
2. Evaporate the solvent under reduced pressure, and purify the crude mixture by column chromatography using EtOAc as an eluent (R_f: 0.2, TLC). The product is a pale yellow liquid. The yield should be ~81 % (1.3 g).

3.1.5 Boc-(2-(2-Methoxyethoxy) Ethyl)-L-Serine Thymine Ethyl Ester (6a)

1. Dissolve thymine acetic acid (0.287 g, 1.56 mmol) in anhydrous DMF (15 mL), followed by sequential addition of DCC (0.324 g, 1.56 mmol) and DhbtOH (0.254 g, 1.56 mmol) while stirring at room temperature.
2. After 1 h, add the solution of compound **5** (0.5 g, 1.32 mmol) in DMF (10 mL) dropwise to the reaction mixture and continue to stir at 50 °C for 24 h.
3. Filter off the pale yellow solid (DCU by-product), and evaporate the filtrate under reduced pressure. Partition the residue with ethyl acetate (100 mL) and saturated aqueous $NaHCO_3$ solution (100 mL). Wash the organic layer with 10 % $KHSO_4$ (50 mL), 10 % $NaHCO_3$ (50 mL), and brine (50 mL) and then dry over anhydrous Na_2SO_4.
4. Evaporate the solvent under reduced pressure, and purify the crude product by silica gel chromatography using EtOH/EtOAc mixture (5/95) as an eluent (R_f: 0.6, TLC). The product is a white foamy solid. The yield should be ~67 % (0.5 g).

Follow the same procedure as described for thymine ester (**6a**) to prepare adenine ester (**6b**), guanine ester (**6c**), and cytosine ester (**6d**).

3.1.6 Boc-(2-(2-Methoxyethoxy) Ethyl)-L-Serine Thymine Monomer (7a)

1. Add 2 M NaOH (15 mL) dropwise over a period of 15 min to a stirred solution of compound **6a** (0.480 g, 0.88 mmol) in THF (15 mL) at 0 °C.
2. After 30 min, dilute the reaction mixture with water (100 mL) and extract with ethyl acetate (2 × 25 mL).
3. Acidify the aqueous layer with 1 M HCl to pH ~4 at 0 °C and then extract with ethyl acetate (3 × 100 mL). Combine the organic layers and dry over anhydrous Na_2SO_4.
4. Evaporate the solvent under reduced pressure and purify by column chromatography using MeOH/DCM (20/80) mixture as an eluent (R_f: 0.4, TLC). The product is an off-white solid. The yield should be ~87 % (0.4 g).

Follow the same procedure as described for thymine monomer (**7a**) to prepare adenine (**7b**), guanine (**7c**), and cytosine (**7d**) monomers.

3.2 A General Procedure for Preparing MTPA Derivatives

1. Add 5 mL of TFA/m-cresol mixture (95:5) to a cold solution of compound **2** (0.10 g, 0.36 mmol) in DCM (5 mL) while stirring.
2. After 30 min, remove the ice bath and allow the reaction mixture to stir at room temperature until all the starting material is consumed (~2 h), as determined by TLC.
3. Evaporate the solvent under reduced pressure, triturate the crude mixture with diethyl ether (3 × 5 mL), dry the resulting residue under high vacuum, and proceed to next coupling step without further purification.
4. Dissolve the above crude residue in DCM (5 mL) and place in an ice bath while stirring. To this reaction mixture, add DIPEA (0.125 mL, 0.72 mmol) followed by (S)-(+)-α-methoxy-α-trifluoromethylphenylacetyl chloride (MTPA-Cl, 0.074 mL, 0.396 mmol), and continue to stir the reaction mixture at room temperature overnight.
5. Dilute the reaction mixture with DCM (20 mL) and wash with water (2 × 10 mL) and then brine. Dry the DCM layer over anhydrous Na_2SO_4, remove the solvent under reduced pressure, and purify the crude mixture by column chromatography using EtOAc as an eluent (R_f: 0.3). The product is a colorless viscous liquid. The yield should be ~70 % over two steps (0.104 g).

Follow the same procedure for preparing compounds **9** and **10**.

3.3 Solid-Phase Synthesis of γPNA Oligomers

Solid-phase synthesis of γPNA oligomers consists of four major steps: (1) resin loading, (2) deprotection, (3) coupling, and (4) cleavage. It is recommended that Kaiser be performed at every few coupling steps to ensure that the reaction proceeds smoothly, with near-quantitative yield in each step.

3.3.1 Resin Swelling (100 mg)

1. Place 100 mg MBHA resin in the reaction vessel (or reactor).
2. Add 2 mL DCM to disperse the resin and let it stand for 1 h.
3. Drain out the solvent and wash with DCM (2×).
4. Wash the resin with 5 % DIEA in DCM followed by DCM (2×).
5. Drain the solvent.

3.3.2 Lysine Coupling

1. Mix **Solutions D** and **E** together and vortex for 1 min to activate the lysine monomer, and then add the mixture to the resin.
2. Agitate the reactor to allow resin mixing for 1 h.
3. Wash the resin with DMF (2×) followed by DCM (4×).
4. Neutralize the remaining free amines with 5 % DIEA/DCM, followed by DCM wash (4×).

3.3.3 Capping

1. Add ~1.5 mL **Capping Solution** to the resin, and agitate the reaction vessel for 30 min.
2. Drain the **Capping Solution**, and repeat **step 2** once more.
3. Drain the **Capping Solution**, and wash the resin with DCM (2×).
4. Perform the Kaiser test.
5. The resin should be colorless (or pale yellow), indicating that all the amino groups have been capped. If not, the resin should be blue. In that case, repeat **steps 1–5** again.

3.3.4 Deprotection

1. Add 1 mL **Deprotection Solution** to the resin, agitate the reaction vessel to induce mixing for 5 min, and then drain the **Deprotection Solution**.
2. Repeat **step 1** again for a total of two rounds of deprotection.
3. Drain the **Deprotection Solution**, and wash the resin with DCM (3×) and then DMF (3×).
4. Perform the Kaiser test. If the test is positive (the resin is blue), repeat **steps 1–3** (*see* **Note 6**).

3.3.5 PNA Coupling

1. Activate the monomer by mixing 300 μL **Solution A** with 150 μL **Solution B** and 150 μL **Solution C**, vortex for 10 s, and then let it stand for 3 min.
2. Add the coupling solution to the resin, and agitate the reaction vessel for 15 min to permit mixing.
3. Drain the coupling solution, and wash the resin thoroughly with DMF (4×) and then DCM (1×).
4. Perform the Kaiser test. If the test is positive (the resin is blue), repeat **steps 1–3**. We never had this problem. It is not necessary to perform the Kaiser test at every coupling step. Do it once every four or five coupling steps once the reaction conditions have been optimized.

3.3.6 Cleavage

1. Remove Boc by adding 1 mL **Deprotection Solution** (m-cresol/TFA: 5/95) to the resin, and agitate the reaction vessel for 4 min. Do this twice.
2. Drain the **Deprotection Solution**.
3. Add the **Cleavage Solution** to the resin, and agitate the reaction vessel to permit mixing for 1 h.
4. Collect the **Cleavage Solution** from the reaction vessel in a 15-mL tube.
5. Add 10 mL ethyl ether to the **Cleavage Solution**, close the cap, and shake the tube briefly. White precipitate should form (γPNA oligomers).
6. Centrifuge the tube at 4,000 cpm for 5 min, and then decant the solvent.
7. Add 10 mL ethyl ether to the tube, vortex thoroughly, and repeat **step 6** once more (washing).
8. Decant the solvent, and air-dry the pellet.
9. Purify the oligomer by RP-HPLC using gradient water/acetonitrile mixtures containing 0.1 % TFA. Verify the identity of the oligomer by MALDI-TOF MS (*see* **Note 7**).

4 Notes

1. Clean and dry all glassware in an oven prior to use.
2. $NaBH_4$ reduction reaction should be carried out in a relatively large flask, because addition of $NaBH_4$ to the filtrate causes strong effervescence.
3. Heating at higher temperatures (above 50 °C) can lead to racemization.
4. Prepare 2,4-dinitrobenzenesulfonyl glycine ethyl ester according to the procedure in ref. 35.
5. Make sure that the result of the Kaiser test is based on the color of the resin and not the color of the solution.
6. A slight excess of the monomer is used in the coupling reaction to maximize the coupling efficiency and minimize formation of tetramethyl guanidine-capped side product. This side product appears as M + 100 in the MALDI-TOF spectrum.
7. Thioanisole and m-cresol are used as scavengers in the cleavage cocktail.

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Chapter 2

Cyclopentane Peptide Nucleic Acids

Ethan A. Englund, Ning Zhang, and Daniel H. Appella

Abstract

Incorporating a cyclopentane ring into the two-carbon unit of a peptide nucleic acid backbone increases its binding affinity to complementary nucleic acid sequences. This approach is a general method to improve binding and can be applied at either purine or pyrimidine bases.

Key words PNA, Cyclopentane, Nucleic acid

1 Introduction

Chemical modification to *aeg*PNA can be used to enhance certain physical properties and may be used to improve applications in biomedical research [1]. Chemically modified peptide nucleic acid (PNA) oligomers may have properties such as increased binding to natural nucleic acids, higher mismatch discrimination, selectivity to bind DNA versus RNA (or vice versa), selectivity between binding parallel and antiparallel nucleic acid sequences, increased solubility, and increased cellular uptake [2]. Using cyclic constraints within the achiral PNA architecture is one method to produce some of the aforementioned benefits [3]. Although the synthesis and characterization of modified *aeg*PNA monomers have usually required a laboratory dedicated to organic synthesis, increased access to commercially available intermediates has made the acquisition of cyclic PNA monomers particularly practical for use in molecular biology research.

(S,S) *trans*-cyclopentane PNA (*tcyp*PNA) (Fig. 1) maintains the basic structure of *aeg*PNA except that its conformation is restrained by a cyclopentane unit in place of the flexible ethylene group [4]. Adding chirality and rotational constraints to PNA (whether in the form of cyclic subunits or substitution along the ring) can predispose PNA oligomers into favorable binding conformations [2, 5]. Because of this, the (S,S) *tcyp*PNA residues increase the binding affinity and mismatch selectivity of PNA probes

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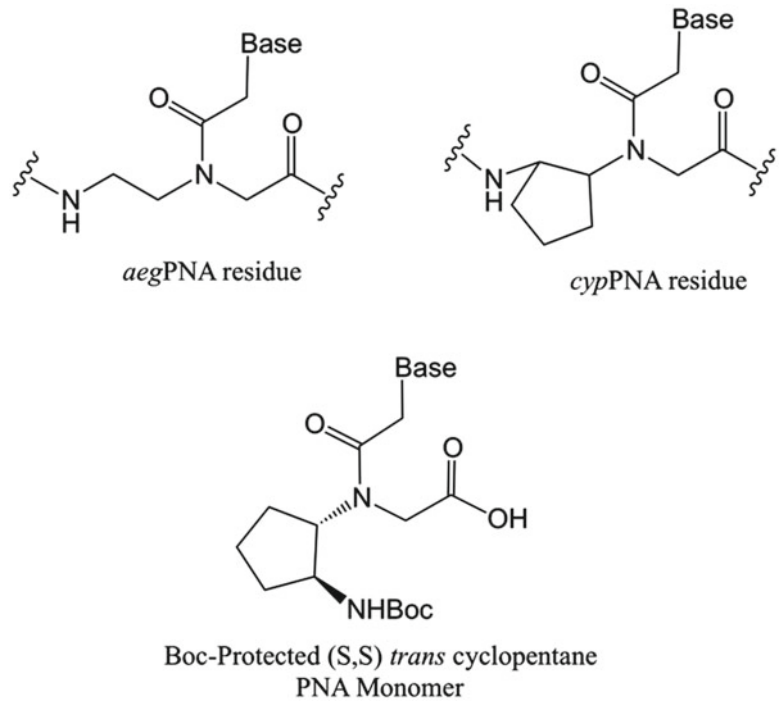

Fig. 1 Chemical structures for a single base unit of aegPNA and tcypPNA

to both single-stranded DNA and RNA (Table 1). This added stability also extends to PNA:DNA complexes beyond the canonical duplex form [6]. In many applications, it would be advantageous to reduce the length of PNA probes without reducing the binding temperature (Table 2). PNA sequences of similar length can vary considerably in binding affinity to complementary nucleic acids based on the ratio of pyrimidine nucleobases to purine nucleobases between different sequences [7]. Being able to selectively increase the binding temperatures of thymine-rich PNA probes could directly benefit diagnostic techniques by helping A:T-rich sequences attain similar stability to G:C-rich sequences (Table 3). Boc-protected (S,S) *tcyp*PNA monomer can be incorporated into *aeg*PNA oligomers using existing methods covered elsewhere in this volume (either by manual or fully automated solid-phase synthesis), so any lab possessing the capability to take advantage of PNA technology should be able to easily incorporate this cyclic derivative into their methodologies.

Recently, one of the key intermediates for the synthesis of (S,S) *tcyp*PNA monomers (the mono-protected enantiomerically pure (1,2)-*trans*-cyclopentane diamine) has become available from a number of vendors. Although protected nucleobase acetic acids are available from vendors, thymine acetic acid is still the least expensive option for the synthesis of large quantities of Boc-protected (S,S)

Table 1
Melting temperature (Tm) of PNA oligomers to single stranded DNA, RNA

PNA sequence[a]	Tm (°C)[b] DNA	Tm (°C)[b] RNA
NH_2-(egl)$_2$-CCT TTG TAC TAT CCA-Lys-$CONH_2$	59.0	69.4
NH_2-(egl)$_2$-CCT TTG T$_{tcyp}$AC T$_{tcyp}$AT CCA-Lys-$CONH_2$	66.7	74.9
NH_2-(egl)$_2$-CCT$_{tcyp}$ TTG T$_{tcyp}$AC T$_{tcyp}$AT CCA-Lys-$CONH_2$	70.0	76.9
NH_2-(egl)$_2$-CCT$_{tcyp}$ T$_{tcyp}$ T$_{tcyp}$G T$_{tcyp}$AC T$_{tcyp}$AT$_{tcyp}$CCA-Lys-$CONH_2$	76.9	Not available

[a] *tcyp* = PNA residue derived from (S,S)-*trans*-1,2-cyclopentane diamine, egl = 8-amino-3,6-dioxaoctanoic acid
[b] Tm represents the melting temperature for the duplex formed between the indicated PNA and antiparallel DNA. Conditions for Tm measurement: 3.0 µM of PNA:DNA duplex, 150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.0, 0.1 mM EDTA, UV measured at 260 nm from 90 to 20 °C, in 1 °C increments. All values are averages from two or more experiments

Table 2
Reduction of PNA oligomer length while maintaining binding temperatures

PNA sequence[a]	Tm (°C)[b] DNA
Biotin-(egl)5- GTC CTG TAG TTC ATT-Lys-CONH2	62.3
Biotin-(egl)5- TC CTG TAG TTC AT-Lys-CONH2	55.6
Biotin-(egl)5- TC CTG T$_{tcyp}$AG TTC AT-Lys-CONH2	60.1
Biotin-(egl)5-CT$_{tcyp}$G T$_{tcyp}$AG T$_{tcyp}$T$_{tcyp}$C A-Lys-CONH2	69.3

[a] *tcyp* = PNA residue derived from (S,S)-*trans*-1,2-cyclopentane diamine, egl = 8-amino-3,6-dioxaoctanoic acid
[b] Tm represents the melting temperature for the duplex formed between the indicated PNA and antiparallel DNA. Conditions for Tm measurement: 3.0 µM of PNA:DNA duplex, 150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.0, 0.1 mM EDTA, UV measured at 260 nm from 90 to 20 °C in 1 °C increments. All values are averages from two or more experiments

Table 3
Modification of *aeg*PNA oligomers to maintain uniform binding characteristics

PNA sequence[a]	Tm (°C)[b] DNA
NH_2-(egl)$_2$-CCT TTG TAC TAT CCA-Lys-$CONH_2$	59.0
Biotin-(egl)5-GTC CTG TAG TTC ATT-Lys-CONH2	62.3
AcNH-TCA TTC GAG TAG CGG-Lys-$CONH_2$	76.5
NH_2-(egl)$_2$-CCT$_{tcyp}$ T$_{tcyp}$T$_{tcyp}$G T$_{tcyp}$AC T$_{tcyp}$AT$_{tcyp}$CCA-Lys-$CONH_2$	76.9
Biotin-(egl)5- GT$_{tcyp}$C CT$_{tcyp}$ G T$_{tycp}$AG T$_{tcyp}$TC ATT-Lys-CONH2	76.4

[a] *tycp* = PNA residue derived from (S,S)-*trans*-1,2-cyclopentance diamine, egl = 8-amino-3,6-dioxaoctanoic acid
[b] Tm represents the melting temperature for the duplex formed between the indicated PNA and antiparallel DNA. Conditions for Tm measurement: 3.0 µm if PNA:DNA duplex, 150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.0, 0.1 mM EDTA, UV measured at 260 nm from 90 to 20 °C, in 1 °C increments. All values are averages from two or more experiments

*tcyp*PNA monomers. This methods review covers the shortened synthesis of this monomer. Although more detailed synthesis and characterization data is readily available, the goal of this methods review is to clearly describe the shortened synthesis in a stepwise manner so that labs that might normally avoid organic chemistry will not be intimidated and perhaps turn to *tcyp*PNA residues if their PNA research could benefit from it.

2 Materials (*See* Note 1)

1. (1*S*,2*S*)-*trans*-*N*-Boc-1,2-cyclopentanediamine can be purchased from either Entrechem Biotechnology or Sigma Aldrich (*see* **Note 2**).
2. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC*HCl) was purchased from Sigma Aldrich.
3. Triethylamine (TEA) was purchased from Sigma Aldrich.
4. Dimethyl formamide (DMF).
5. Lithium hydroxide (LiOH) was purchased from Sigma Aldrich.
6. Sodium bicarbonate ($NaHCO_3$) was purchased from Sigma Aldrich.
7. Sodium sulfate (Na_2SO_4) was purchased from Sigma Aldrich.
8. Ethyl acetate (EtOAc) was purchased from Sigma Aldrich.
9. 4-Dimethylaminopyridine (4-DMAP) was purchased from Sigma Aldrich.
10. Tetrahydrofuran (THF).

3 Methods (*See* Note 3)

1. Boc-protected (S,S) *trans* cyclopentane diamine (1.5 g, 7.6 mmol) is dissolved in 150 mL of dry DMF in a 250 mL round-bottom flask, stirring under N_2.
2. TEA (1.1 mL, 7.6 mmol) and methyl bromoacetate (0.6 mL, 6.8 mmol) are added dropwise via a syringe (*see* **Note 4**).
3. The reaction is stirred at room temperature for 3 h.
4. The reaction is diluted with 50 mL of saturated aqueous $NaHCO_3$.
5. The combined mixture is extracted with EtOAc (twice, 35 mL).
6. The combined organic layers are washed with saturated NaCl solution (twice, 25 mL), dried over Na_2SO_4, and concentrated under reduced pressure.

7. The crude residue is purified via flash column chromatography [Rf = 0.30 (EtOAc)] to yield *tcyp*PNA backbone as a colorless oil, which forms a colorless crystalline solid at room temperature. (The yield should be about 68 % or 1.26 g.)
8. The purified *tcyp*PNA backbone (1.26 g, 4.6 mmol) is dissolved in 25 mL of dry DMF in a 50 mL round-bottom flask.
9. The solution is put under N_2 and cooled to 0 °C via an ice bath.
10. Thymine acetic acid (1.27 g, 6.9 mmol) and DMAP (150 mg, 1.2 mmol) are added.
11. EDC*HCl (1.76 g, 9.2 mmol) is added to the solution which is allowed to stir for 10 min at 0 °C.
12. The ice bath is removed, and the solution is stirred for an additional 36 h.
13. The solution is added to H_2O (150 mL) and extracted with EtOAc (three times, 100 mL).
14. The combined organic layers are washed with saturated NaCl (four times, 125 mL), dried over Na_2SO_4, concentrated under reduced pressure, and dried under vacuum to yield the Boc-protected (S,S) *tcyp*PNA monomer methyl ester (thymine) as a colorless solid. If necessary, the solid was purified by flash column chromatography: $R_f = 0.21$ (1 % MeOH/EtOAc) (expected yield about 87 % or 1.75 g).
15. The 1.75 g of Boc-protected (S,S) *tcyp*PNA monomer methyl ester (thymine (4.0 mmol)) is dissolved in 63 mL of THF and cooled to 0 °C via ice bath.
16. Lithium hydroxide monohydrate is dissolved in 53 mL H_2O and added to the stirring solution slowly over 5 min.
17. Ice bath is removed, and the solution is stirred at room temperature for 5 h.
18. Dilute the mixture with 86 mL H_2O, and wash the aqueous solution with 95 mL of diethyl ether three times.
19. The aqueous layer is acidified with aqueous 3 N HCl to pH 1 (*see* **Note 3**).
20. The solution is extracted with 133 mL of EtOAc five times.
21. The combined organic layers are dried over Na_2SO_4, concentrated under reduced pressure, and dried under vacuum to yield the Boc-protected (S,S) *tcyp*PNA monomer (thymine) as a colorless solid. (The approximate yield should be about 96 % or 1.63 g.)

4 Notes

1. The equipment needed for this procedure is common to an organic chemistry lab. A stirring plate, round-bottom flasks, separatory funnels, a rotary evaporator, and silica packed columns. Characterization data can be acquired for each intermediate in the synthesis and can be matched to the full characterization data available in ref. 4.
2. If using cyclopentane diamine purchased from Sigma Aldrich, our lab found it useful to recrystallize the diamine before synthesis to increase the enantiomeric purity.
3. The procedure as described has been adapted from ref. 4. The procedure as described should result in about 1.6 g or 3.8 mmol of Boc-protected (S,S) *tcyp*PNA monomer (thymine). The reaction can easily be scaled up or down depending on the amount of PNA monomer needed for study.
4. It is very important that methyl bromo acetate is added slowly to the solution. Increasing the concentration too quickly will result in a large amount of dialkylated side product. It is for this reason also that the methyl bromo acetate is the limiting reagent in the reaction.

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Chapter 3

Chiral PNAs with Constrained Open-Chain Backbones

Roberto Corradini, Tullia Tedeschi, Stefano Sforza, and Rosangela Marchelli

Abstract

Chiral open-chain PNAs have been shown to have improved properties in terms of control of helical handedness, DNA affinity, sequence selectivity, and cellular uptake. They can be synthesized either using preformed chiral monomers or by means of a submonomeric strategy. The former is preferred when only a stereogenic center is present at C-5, whereas for PNA-bearing substituents at C-2, the submonomeric approach is preferred, since racemization, generally occurring during the solid-phase synthesis, can be minimized by this procedure. Here we describe the protocols for the synthesis of PNA oligomers containing C-2- or C-5- (or both) modified monomers and a GC method for checking the optical purity of C-2-modified PNAs.

Key words Stereochemistry, Chiral PNA, Submonomeric strategy, Optical purity, Solid-phase synthesis

Acronym List

BTSA	*N*,*O*-Bis(trimethylsilyl)acetamide
CMB	Carboxymethyl nucleobase
DCC	*N*,*N*-dicyclohexylcarbodiimide
DCM	Dichloromethane
DIC	*N*,*N*-diisopropylcarbodiimide
DIPEA	*N*,*N*-diisopropylethylamine
DhBTOH	3-hydroxy-1,2,3-benzotriazin-4(3H)-one
DMF	*N*,*N*-dimethylformamide
EDC	*N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride
HBTU	*O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate
HOBt	1-hydroxy-1,2,3-benzotriazole
MBHA	4-methyl benzhydrylamine
NMP	N-methylpyrrolidone
TFA	Trifluoroacetic acid
TFMSA	Trifluoromethanesulfonic acid

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1 Introduction

Chiral open-chain PNAs have been shown to have improved properties in terms of control of helical handedness, DNA affinity, sequence selectivity, and cellular uptake. They can be synthesized either using preformed chiral monomers or by means of a submonomeric strategy. The former is preferred when only a stereogenic center is present at C-5, whereas for PNA bearing a stereogenic center at C-2, the submonomeric approach is preferred, since racemization, generally occurring during the solid-phase synthesis, can be minimized by this procedure. Here we describe the protocols for the synthesis of PNA oligomers containing C-2- or C-5- (or both) modified monomers and a GC method for checking the optical purity of C-2-modified PNAs.

Many modifications of the basic PNA structure have been proposed in order to improve the performance of these DNA analogues in terms of affinity and specificity towards complementary oligonucleotide sequences. Actually many of them involved the presence of one or more stereogenic centers, allowing to study the effect of chirality on the PNA preferred handedness and, as a consequence, on DNA recognition [1].

Chiral PNA derivatives can be divided into two general classes (Fig. 1): (a) flexible, acyclic PNA molecules, with side chains derived from amino acids (such as lysine or alanine), and (b) highly

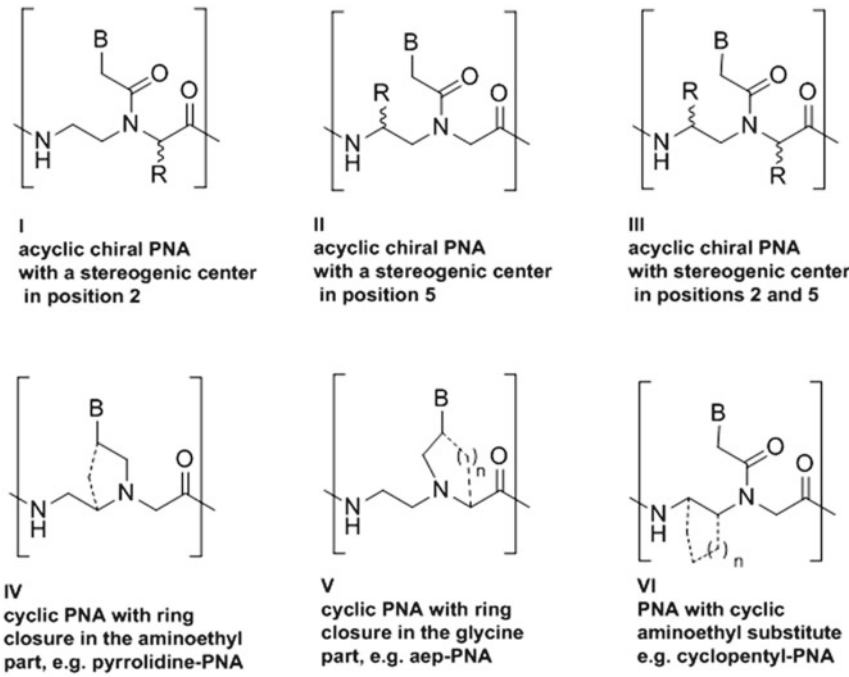

Fig. 1 Different types of chiral PNA derivatives with acyclic and cyclic backbones

constrained cyclic PNA oligomers, which can be obtained from proline or from cyclohexane or cyclopentane derivatives.

In this chapter we focus on the use of chiral open-chain PNA including one or more modified units of type I–III monomers. Chiral monomers of type I were first described by Nielsen and collaborators [2–4] and then widely used by our group [5, 6]. Type II PNAs were described for the first time by Liang [8] and then later on used by others using positively charged side chains [9–12]. The combination of these two models led us to propose the type III PNAs as new entities [13, 14].

The introduction of stereogenic centers bearing side chains into the PNA backbone affords the possibility to modulate the PNA properties at different levels: (a) control of the DNA affinity by additional interactions provided by the side chains; (b) stereochemical bias affecting the ability to form helical structures; (c) selectivity in the DNA/RNA binding, in particular direction control (antiparallel vs. parallel orientation) and mismatch selectivity; and (d) increase of cellular permeability.

1.1 Effect of Stereochemistry on Helical Preference

The effect of stereochemistry and of the side chains on DNA binding ability can hardly be predicted. Although, as a general rule, the presence of a substituent either in positon-2 or -5 creates a steric hindrance with the neighboring groups, thus inducing a decrease in the PNA–DNA stability, this effect can be counterbalanced by the electrostatic attractive interactions between DNA and the positively charged side chains on PNA, such as those derived from lysine or arginine synthons, and by the pre-organization of the PNA into a preferred helical structure, thus minimizing the entropy loss upon binding to DNA or RNA and eventually improving the duplex stability.

The effects on the helical induction are stronger if the stereogenic center(s) is placed in the middle of the PNA strands [5, 6], whereas if the chiral monomers are close to the ends of PNA, they are usually quite modest.

The effect of stereochemistry on the helical induction in PNA strands has been systematically studied in the case of acyclic chiral PNAs containing lysine units placed in the middle of the PNA strand. They generally show a preference for either left-handed or right-handed helices according to the configuration(s) of the stereogenic center(s) inserted (*see* **Note 1**).

The effects of the preferred helical handedness for each type of substitution are reported in Table 1 [14].

As one can see, the melting temperature (Tm) of the PNA:DNA duplex with a PNA modified at C-2 with a D-stereogenic center is higher than that observed for the duplex involving the achiral PNA but even higher with the PNA modified at C-5 with a L-stereogenic center and with the 2D,5L-modified PNA. All three modified PNAs have preference for forming righ-handed helices, as evaluated from handedness of PNA:PNA duplexes. On the contrary the

Table 1
Correlation between induction of helicity and DNA binding ability induced by the presence of a single chiral PNA monomer substituted at C-2, C-5, or at both positions

Monomer stereochemistry	Helicity induction by C-2	Helicity induction by C-5	PNA:PNA helical preference (strength of induction)[a]	PNA:DNA Tm (°C)
2D,5L	Right-handed	Right-handed	Right-handed (+++)	57
5L	–	Right-handed	Right-handed (++)	56
2L,5L	Left-handed	Right-handed	Right-handed (+)	52
2D	Right-handed	–	Right-handed (+)	52
Achiral	–	–	None	50
2L	Left-handed	–	Left-handed (–)	47
2D,5D	Right-handed	Left-handed	Left-handed (–)	33
5D	–	Left-handed	Left-handed (––)	32
2L,5D	Left-handed	Left-handed	Left-handed (–––)	<20

(PNA sequence: GTAGAT$_{Lys}$*CACT, where T$_{Lys}$* denotes a Lys-based modified PNA monomer)
[a]Helical preference obtained assigning (++) to induction by the 5L and (+) to induction by the 2D-substituent; (––) to induction by the 5D and (–) to induction by the 2L-substituent

2-L-, the 5-D-, and the 2L,5D-modified PNAs, which give rise to duplexes with DNA with lower Tm, have a strong preference for left-handedness. On the base of this study the preferred handedness of PNA strands containing acyclic chiral PNA monomers can be predicted: for 2D- or 5L- or 2D,5L-PNA the right-handedness is preferred; for 2L or 5D or 2L,5D left-handedness is preferred; for 2L,5L there is a chiral conflict, but the induction exerted by the stereocenter in position 5 is stronger, and thus right-handedness is preferred; for 2D,5D there is also a chiral conflict, but the induction exerted by the stereocenter in position 5 is stronger, and thus left-handedness is preferred.

1.2 Effect of Stereochemistry on DNA Binding and Selectivity

The preference for a given helical handedness, in turn, affects the affinity of the binding to nucleic acids (DNA or RNA), according to the principle that, since DNA and RNA generally adopt right-handed conformations, PNAs with a preference for right-handedness are generally favored in binding DNA and RNA [13, 14] (Table 1).

Other factors also play important roles (such as the presence of a positive or a negative charge in the backbone as well as steric hindrance) so that the overall stability of the chiral PNA–nucleic acid complex will emerge as a delicate balance of these factors. However, when considering two enantiomeric PNAs, the one preferring a right-handed helical conformation will bind complementary nucleic acids better than its enantiomer, with a difference which will be strictly related to the strength of the helical induction in the PNA strand [14] (*see* **Note 2**).