A Simple γ-Backbone Modification Preorganizes Peptide Nucleic Acid into a Helical Structure

Anca Dragulescu-Andrasi, Srinivas Rapireddy, Brian M. Frezza, Chakicherla Gayathri, Roberto R. Gil,* Danith H. Ly*

Department of Chemistry, Carnegie Mellon University, 4400 Fifth Ave., Pittsburgh, Pennsylvania 15213

Materials and Procedures

Abbreviations:

N-Methyl morpholine (NMM); Dichloromethane (DCM); N,N-diisopropylethylamine (DIPEA); N-Methyl-2-pyrrrolidone (NMP); O-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU); N-Methylidicyclohexylamine (MDCHA); N,N-Dimethylformamide (DMF); O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU); Acetic anhydride (Ac2O); trifluoromethanesulfonic acid (TFMSA); Trifluoroacetic acid (TFA); Bromo-tris-pyrrrolidino phosphoniumhexafluorophosphate (PyBrop); Ethyl acetate (EtOAc); 3-hydroxy-1,2,3-benzotriazin-4(3H)-one (DhbtOH); Hexane (Hex); Methanol (MeOH); Acetic acid (AcOH); Tetrahydrofuran (THF); Ethanol (EtOH); Acetonitrile (ACN); High Pressure Liquid Chromatography (HPLC); Matrix-Assisted Laser Desorption Time-of-Flight (MALDI-TOF); Thin Layer Chromatography (TLC).

Materials and equipment:

Solvents were dried by standard methods and freshly distilled prior to use. Commercial reagents were used without further purification. Boc-Ser(Bzl)OH and PyBrop were purchased from Novabiochem. All other chemicals were purchased from Aldrich. 1H-NMR spectra were acquired on a Bruker Avance AV-300 NMR spectrometer using standard Bruker software. Flash chromatography was performed using scientific silica gel (32-63) from Selecto Scientific. TLC was performed with silica gel 60 F254 plates (Merck). Mass spectra were measured on Finnigan LCR ESI / APCI ion trap mass spectrometer by electro spray ionization. MALDI-TOF experiments were carried out on an Applied Biosystems Voyager-DE™ STR Biospectrometry™ Workstation. 10mg/mL solution of α-hydroxycinnamic acid in ACN-water (1:1) with 0.1%TFA was used for generating the probe-matrix mixture. CD experiments were performed on a Jasco J-715 spectropolarimeter equipped with a thermoelectrically-controlled single-cell holder. UV-Vis measurements were performed on a Varian Cary 300 Bio spectrophotometer equipped with a thermoelectrically controlled multicell holder.

Boc/Z-protected PNA monomers were purchased from Applied Biosystems. PNA and 1S-γPNA oligomers were synthesized using standard solid-phase synthesis protocols. The oligomers were purified using reversed-phase HPLC and characterized by MALDI-TOF. All PNA and 1S-γPNA stock solutions were prepared in nanopure water. The concentrations were determined...
at 85 °C using the following extinction coefficients for PNA monomers: 6600 M⁻¹ cm⁻¹ (C), 8600 M⁻¹ cm⁻¹ (T), 13 700 M⁻¹ cm⁻¹ (A), 11 700 M⁻¹ cm⁻¹ (G). DNA oligomers were purchased from Integrated DNA Technologies, Inc. as lyophilized powders and used without further purification.

**Circular Dichroism (CD) analysis:**
Samples were prepared in buffer containing 10 mM sodium phosphate (pH 7.0), 1 mM EDTA, and 100 mM NaCl. All spectra represent an average of at least ten scans collected at 100 nm/min rate between 200-320 nm, in a 1-cm path-length cuvette at 22 °C. CD spectrum from buffer solution was subtracted from the sample spectra, which were then smoothed via a five-point adjacent averaging algorithm.

**Thermal denaturation analysis:**
Samples were prepared in the same buffer used in CD experiments above. Unless otherwise indicated, samples were heated to 90 °C and equilibrated for 10 min. UV-Vis absorbance at 260 nm was recorded every 0.5 °C as the samples were cooled and then heated at a rate of 1.0 °C/min. All experiments were performed in triplicate.

![Scheme S1. Synthesis of \(^1\)S-\(\gamma\)-PNA monomers. Reagents and conditions: (a) NMM, ClCO₂iBu, CH₃NHOCH₃·HCl, CH₂Cl₂, rt, 14 h (97%), (b) LiAlH₄, THF, 0 °C, 1 h (95%), (c) ethyl ester glycine, MeOH, 4 °C, 4 h, then AcOH, NaBH₃CN, 30 min (68%), (d) carboxymethylnucleobase, DCC, DhbtOH, DMF, 40°C, 24 h (62%), (e) 1 M LiOH, THF, 0 °C 30 min (95%).](image)

**Synthesis of \(^1\)S-\(\gamma\)PNA monomers:**

\(N\)-tert-butyloxycarbonyl-\(O\)-benzyl serine-weinrebamide 2: To a stirred solution of 1 (5 g, 16.9 mmol) in anhydrous DCM (100 mL) was added NMM (4.1 mL, 37.2 mmol) at -15 °C (dried ice/MeOH) under argon atmosphere. Isobutyl chloroformate (2.45 mL 18.75 mmol) was added drop-wise. After stirring for 15 min, \(N,O\)-dimethylhydroxylamine hydrochloride (1.64 g, 16.9 mmol) was added and the stirring was continued for 14 h at room temperature. The solution was washed with 0.2 M KH₂SO₄ (60 mL) and the aqueous layer extracted with DCM (3 x 50 mL). The combined organic phases were dried (MgSO₄) and the solvent was removed under reduced
The residue was purified by silica gel flash chromatography to afford 5.55 g (97%) of 2 as colorless solid. \( R_t = 0.5 \) (Hex:EtOAc = 2:3). \(^1\)H NMR (300 MHz, CDCl\(_3\)) 7.29 (5H, m, -Ph-H), 5.42 (1H, br d, J = 8.4 Hz, Boc-NH-), 4.88 (1H, br dt, J = 8.4 and 4.8 Hz, -NH-CH-C-), 4.53 (2H, AB System, J = 12.2 Hz, -O-CH\(_2\)-Ph), 3.71 (3H, s, -O-CH\(_3\)), 3.67 (AB part of ABX system, J = 9.5 and 4.8 Hz, -CH-CH\(_2\)-O-), 3.21 (3H, s, -N-CH\(_3\)), 1.44 (9H, s, t-butyl-CH\(_3\)). ESI-MS (m/z): Mass calculated for C\(_{17}\)H\(_{26}\)O\(_5\)N\(_2\)H\(_2\) 338.18, found 361.00 (338.01 + Na\(^+\)).

\( \text{N-tert-butyloxycarbonyl-}O\text{-benzyl serinal 3:} \) A stirred solution of 2 (2.3 g, 6.78 mmol) in dry THF (60 mL) was cooled to 0 °C under argon atmosphere. Lithium aluminum hydride (0.5 g, 13.6 mmol) was added in portions and after 1 h, 0.2 M KHSO\(_4\) (30 mL) was added. The organic compounds were extracted with diethyl ether (3 x 50 mL). The combined organic layers were washed with 1 M HCl (3 x 15 mL), brine (3 x 15 mL) and dried (MgSO\(_4\)). The solvent was removed under reduced pressure to afford 1.8 g (95%) of 3 as yellow oil, which was used in the next step without further purification. \( R_f = 0.8 \) (Hex:EtOAc, 2:3).

\( \text{N-tert-butyloxycarbonyl-1-(O-benzylxomethyl) – aminoethylglycine ethyl ester 4:} \) A stirred solution of 3 (1.8 g, 6.45 mmol) in dry MeOH (50 mL) was cooled to 0 °C under argon atmosphere. In a separate round bottom flask, glycine ethyl ester hydrochloride (1.89 g, 13.56 mmol) was dissolved in MeOH (5 mL) and DIPEA (2.36 mL, 13.56 mmol). The flasks were then mixed and stirred at 4 °C for 4 h at which point point acetic acid (1.2 mL, 20 mmol) was added, followed by NaBH\(_3\)CN (0.62 g, 10 mmol) and the solution was allowed to stir for another 30 min. 10% NaHCO\(_3\) (100 mL) was then added to the solution and extracted with EtOAc (3 x 30 mL). The combined organic layers were washed with brine and dried (MgSO\(_4\)). The solvent was evaporated under reduced pressure and the residue was purified by flash silica gel chromatography to afford 1.6 g (68%) of 4 as viscous liquid. \( R_t = 0.38 \) (Hex:EtOAc = 2:3). \(^1\)H NMR (300 MHz, CDCl\(_3\)) 7.31 (5H, m, -Ph-H), 5.10 (1H, br s, Boc-NH-), 4.52 (2H, s, -O-CH\(_2\)-Ph), 4.18 (2H, q, 7.2 Hz, -O-CH\(_2\)-CH\(_3\)), 3.82 (1H, dd, 9.4 Hz and 3.7 Hz, -CH-CH\(_2\)-O-), 3.52 (1H, dd, 9.4 Hz and 4.9 Hz, -CH-CH\(_2\)-O-), 3.4 (2H, AB System, 17.3 Hz, -NH-CH\(_2\)-C-), 2.87 (1H, dd, 12.2 Hz and 5.9 Hz, -CH-CH\(_2\)-NH-), 2.77 (1H, dd, 12.2 Hz and 5.7 Hz, -CH-CH\(_2\)-NH-), 1.44 (9H, s, t-butyl-CH\(_3\)), 1.27 (3H, t, 7.2 Hz, -O-CH\(_2\)-CH\(_3\)). ESI-MS (m/z): Mass calculated for C\(_{19}\)H\(_{30}\)O\(_5\)N\(_2\) 366.45, found 366.93.

\( \text{Boc-(BzlOCH\(_2\))T-OEt 5a:} \) To a stirred solution of thymine acetic acid (0.96 g, 5.28 mmol) in dry DMF (20 mL) were added DCC (1.08 g, 5.28 mmol) and DhbtOH (0.85 g, 5.28 mmol) under argon atmosphere. The stirring was continued for 1 h at room temperature. Compound 4 (1.6 g, 4.4 mmol) in dry DMF (5 mL) was added and the reaction was stirred at 50 °C for 24 h. Following evaporation of the solvent, the residue was partitioned between EtOAc (20 mL) and saturated NaHCO\(_3\) (100 mL). The organic layer was washed with 10% KHSO\(_4\) (3 x 50 mL), 10% NaHCO\(_3\) (3 x 50 mL), brine (10 mL) and dried over MgSO\(_4\). The solvent was removed under reduced pressure and purified by flash silica gel chromatography to afford 1.43 g (62%) of 5a as pale yellow powder. \( R_t = 0.4 \) (Hex:EtOAc = 1:9). \(^1\)H NMR (300 MHz, CDCl\(_3\)) 8.17 (1H, br s, -COH\(_2\)-NH-), 7.32 (5H, m, -Ph-H), 7.01 & 6.86 (1H, br q, J=1.2 Hz, -N-CH-C-CH\(_3\)), 5.20 & 5.09 (1H, br s, Boc-NH-)*, 4.58 (2H, s, -N-CH\(_2\)-CO-), 4.4-4.7 (2H, m, -O-CH\(_2\)-Ph), 4.16 (4H, m, -O-CH\(_2\)-CH\(_3\) & -CH-CH\(_2\)-NH-), 3.97 & 3.91 (2H, s, -CO-CH\(_2\)-Thymine)*, 3.80 (1H, m, -NH-CH-C-), 3.65-3.45 (2H, m, -CH-CH\(_2\)-O-), 1.90 (3H, br d, J=1.2 Hz, Thymine -CH\(_3\)), 1.43 (9H, s, t-butyl-CH\(_3\)), 1.33-1.20 (3H, t, J=7.1 Hz, -O-CH\(_2\)-CH\(_3\)). ESI-MS (m/z): Mass calculated for C\(_{26}\)H\(_{36}\)O\(_8\)N\(_4\) 531.58, found 555.07 (532.01 + Na\(^+\)).

---

3
Boc-(BzlOCH₂)-T-OH 6a: To a solution of 5a (1.3 g, 2.46 mmol) in THF (20 mL) was added drop-wise 1 N lithium hydroxide (20 mL) at 0 °C. After stirring for 30 min at the same temperature, water (100 mL) was added and the pH was adjusted to 4 at which point precipitate was formed. Filtered the precipitate and washed with cold water and dried under vacuum to yield 1.17g (95%). Rf = 0.1 (EtOAc:EtOH = 4:1). 1H NMR (300 MHz, CDCl₃) δ 8.06 (1H, br s, -CO-NH-CO-), 7.35 (5H, m, -Ph-H), 7.12 & 6.90 (1H, m, -N-CH₂-C₂H₃), 5.20 (1H, br s, Boc-NH-), 4.60 (2H, s, -N-CH₂-CO-), 4.50 (2H, m, -O-CH₂-Ph), 4.16 (2H, m, -CH₂-CH₂-NH-), 3.97 (2H, m, -CO-CH₃), Thymine), 3.80 (1H, m, -NH-CH₂-CH₂-), 3.57 (2H, m, -CH₂-CH₂-O-), 1.90 (3H, m, Thymine –CH₃), 1.43 (9H, s, t-butyl-CH₃). ESI-MS (m/z): Mass calculated for C₂₄O₈N₄H₃₂ 504.53, found 503.00.

Boc-(BzlOCH₂)-GCbz–OH 6b: Guanine monomer synthesis was performed using the same procedure outlined above for thymine monomer. 1H NMR (300 MHz, DMSO) δ 11.37 (1H, s, -CH₂-CO-OH), 7.78 & 7.76 (1H, s, Guanine CH), 7.40-7.25 (10H, m, -Ph-H), 5.26 & 5.24 (2H, s, -CO-O-CH₂-Ph), 4.54 & 4.43 (2H, s, -N-CO-CH₂-Guanine(Z)), 4.0-3.7 (4H, m, -N-CH₂-COOH & -CH₂-O-CH₂-Ph), 1.37 (9H, s, t-butyl-CH₃), Peaks for –NH-CH₂-CH₂- and -CH-CH₂-N₃ are obscured by a broad water peak from 3.7-3.1 due to water absorption from the DMSO. ESI-MS (m/z): Mass calculated for C₃₂O₉N₇H₃₇ 663.68, found 664.20.

Boc-(BzlOCH₂)-CCbz–OH 6c: Cytosine monomer synthesis was performed using the same procedure outlined above. 1H NMR (300 MHz, DMSO) δ 10.74 (1H, br s, -CH₂-CO-OH), 7.84 (1H, d, J=6.9 Hz, Cytosine –CH-CH₂-N-CO), 7.49-7.26 (10H, m, -Ph-H), 5.20 & 5.12 (2H, s, -CO-O-CH₂-Ph), 4.57 & 4.44 (2H, m, -N-CH₂-COOH & -CH₂-O-CH₂-Ph), 1.37 (9H, s, t-butyl-CH₃), Peaks for –NH-CH₂-CH₂- and -CH-CH₂-N₃ are obscured by a broad water peak from 3.7-3.1 due to water absorption from the DMSO. ESI-MS (m/z): Mass calculated for C₃₁O₉N₅H₃₇ 623.65, found 624.52.

Boc-(BzlOCH₂)-ACbz–OH 6d: Adenine monomer synthesis was performed using the same procedure outlined above. 1H NMR (300 MHz DMSO) δ 10.63 (1H, br s, -CH₂-CO-OH), 8.59 & 8.57 (1H, s, Adenine C₄N₂H), 8.30 & 8.26 (1H, s, Adenine C₅N₂H), 7.49-7.26 (10H, m, -Ph-H), 5.22 & 5.12 (2H, s, -CO-O-CH₂-Ph), 4.57 & 4.44 (2H, m, -N-CH₂-COOH & -CH₂-O-CH₂-Ph), 1.37 (9H, s, t-butyl-CH₃), Peaks for –NH-CH₂-CH₂- and -CH-CH₂-N₃ are obscured by a broad water peak from 3.7-3.1 due to water absorption from the DMSO. ESI-MS (m/z): Mass calculated for C₃₂O₈N₇H₃₇ 647.68, found 648.27.

Oligomer synthesis:
PNA and 1S-2PNA oligomers were synthesized manually on L-lysine-substituted methyl benzhydryl amine (MBHA) resin (0.28 meq/g) by standard solid-phase peptide synthesis methods. The MBHA resin (1.0 g) was down-loaded to 0.1 mmol/g with Boc-Lys-(2-Cl-Z)-OH or with the first monomers for the 1S-2PNA oligomers (P6 to P8 and P15 to P18) at lower scale (100 mg resin). The resin was first swelled in DCM for 1 h. The following solutions were prepared: 0.2 M Boc-Lys-(2-Cl-Z)-OH in NMP (A), 0.2 M HATU in NMP (B), and 0.5 M DIEA in NMP (C). These solutions were then combined appropriately to give two additional solutions: 0.45 mL of A + 0.46 mL of C + 1.59 mL NMP (Solution I), and 0.55 mL of B + 1.95 mL NMP (Solution II). Solutions I and II were pre-mixed for 1 min and then added to the resin. The resin*1 to 2 mixed rotameric species in solution
was agitated with a mechanical shaker for 1 h and then drained using air pressure. The resin was subsequently washed with DMF (4x), DCM (4x), 5% DIEA in DCM (1x), and again with DCM (4x). The remaining active sites were then capped with a 1:2:2 solution of Ac₂O:NMP:pyridine (2x, 20 min), followed by DMF (2x) and DCM (4x) washes and a qualitative Kaiser test [1 drop of Kaiser A (980 µL pyridine, 100 µL phenol solution—8g of phenol dissolved in 2 mL EtOH, 20 µL KCN solution — 13 mg KCN in 20 mL water) and 1 drop of Kaiser B (5% w/v ninhydrin in EtOH)] to confirm that all the unreacted primary amines were capped. The resin was then washed with DCM (2x) and allowed to dry under vacuum. The loaded resin was stored at -20 °C for further use.

The following is a representative coupling cycle for one PNA or 14S-γPNA monomer. The loaded resin (100 mg) was swelled in DCM for 1 h. The solvent was drained using positive air pressure and a solution of 5% m-cresol in TFA was added to the resins. The resin was shaken for 4 min, and the solution was removed using positive air pressure. The TFA deprotection was repeated 3 times for deprotection of the lysine residue (or the first 14S-γPNAs) and then twice for every subsequent monomer. This was followed by subsequent washes with DCM (2x), DMF (2x), DCM (2x), and pyridine (1x). Kaiser test was performed to verify the effectiveness of deprotection. After positive Kaiser test, 150 µL of 0.2 M monomer solution in NMP was pre-mixed with 150 µL of 0.8 M MDCHA in pyridine and 300 µL of 0.1 M HBTU in DMF for 1 min and then added immediately to the resin. The resin was shaken for 30 min (1 h for 14S-γPNA monomers). Following coupling, the solution was removed using positive air pressure and the resin was washed with DMF, 5% DIEA/DCM (1x) and DCM (2x). Once more, a qualitative Kaiser test was performed and, if negative, the resin was capped with a 1:25:25 mixture of Ac₂O: NMP: pyridine (2 x 2 min). The capping step was followed by washes with DCM (1x), 20% piperidine/DMF (1x) and then DCM (3x). This cycle was then repeated until the last monomer was coupled.

The oligomers were cleaved from the resin using a TFA/TFMSA/m-cresol/thioanisole mixture (6:2:1:1), and then precipitated with ethyl ether (4x) and air-dried. The oligomers were purified by reverse-phase HPLC and characterized by MALDI-TOF mass spectrometry.
**Figure S1.** HPLC trace of the crude P5 oligomer. Eluent A: 0.1% TFA in water and eluent B: 0.1% TFA in ACN. The linear gradient was 0-10% of eluent B in 40 min at 40 °C at a flow rate of 8.0 mL/min.

**Figure S2.** MALDI-TOF spectrum of the purified P5 oligomer (calculated mass: 3187, observed 3187.9 and 3277.3 (M+4 Na+)).

**Figure S3.** CD spectra of single strand L-S-γPNA oligomers (P5 and P15) in sodium phosphate buffer at 5.0 μM strand concentration each, recorded at 22 °C.
Figure S4. Double Quantum Filtered Phase Sensitive COSY of (C^l-T) L-Ser-γPNA in D$_2$O at 500 MHz.
**Figure S5.** UV-melting curves of the fully-matched PNA- and (^4S-γPNA)-DNA hybrid duplexes. Thermal denaturation studies were performed on a Varian Cary 300 Bio spectrophotometer equipped with thermoelectrically controlled multicell holder, using 2 µM strand concentration each in 100 mM NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7. Thermal denaturation was monitored at 260 nm at the heating and cooling rate of 1 °C/min from 20 to 90 °C. The melting transitions were determined from the first derivatives of the UV-melting curves.

**Figure S6.** UV-melting curves of the fully-matched PNA- and (^4S-γPNA)-RNA hybrid duplexes. Thermal denaturation studies were performed on a Varian Cary 300 Bio spectrophotometer equipped with thermoelectrically controlled multicell holder, using 2 µM strand concentration each in 100 mM NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7. Thermal denaturation was monitored at 260 nm at the heating and cooling rate of 1 °C/min from 20 to 90 °C. The melting transitions were determined from the first derivatives of the UV-melting curves.
Figure S7. UV-melting curves of P5-DNA hybrid duplexes containing perfectly-matched and single-base mismatched sequences. Thermal denaturation studies were performed on a Varian Cary 300 Bio spectrophotometer equipped with thermoelectrically controlled multicell holder, using 2 µM strand concentration each in 100 mM NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7. Thermal denaturation was monitored at 260 nm at the heating and cooling rate of 1 °C/min from 20 to 95 °C. The melting transitions were determined from the first derivatives of the UV-melting curves.

Figure S8. UV-melting curves of P5-RNA hybrid duplexes containing perfectly-matched and single-base mismatched sequences. Thermal denaturation studies were performed on a Varian Cary 300 Bio spectrophotometer equipped with thermoelectrically controlled multicell holder, using 2 µM strand concentration each in 100 mM NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7. Thermal denaturation was monitored at 260 nm at the heating and cooling rate of 1 °C/min from 20 to 90 °C. The melting transitions were determined from the first derivatives of the UV-melting curves.
Figure S9. Effect of nucleobase sequence at the adjacent, N-terminal position (with respect to the position of the chiral unit) on the helical folding of the PNA oligomer.