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Pyrrolidine Carbamate Nucleic Acids: Synthesis and DNA Binding Studies

Meena and Vaijayanti A. Kumar*

Division of Organic Chemistry (Synthesis), National Chemical Laboratory, Pune 411008, India

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Abstract—An efficient solid phase synthesis of pyrrolidine carbamate nucleic acids is reported. The protected (2*S*, 4*S*)-4-amino-pyrrolidine-2-methanol with nucleobases thymine and cytosine attached to the ring nitrogen through an acetyl linker can be activated as nitrophenyl carbonates for the synthesis of dimer, trimer and oligomers.

Introduction

A variety of nucleic acid analogues based on uncharged or partially charged backbone show improved cellular uptake and resistance to degradation by nucleases.[1] Amongst these non-ionic modifications, the carbamate-linked nucleic acid analogues were the earliest reported[2] but are not yet fully exploited for their application as antisense molecules for the control of gene expression. There are reports in the literature describing the synthesis and characterization of oligonucleotides with a 3′-*O*-CO-NH-5′[3a] and 3′-NH-CO-*O*-5′[3b] (Fig. 1a,b) linked 2′-deoxysugars to replace phosphodiester linkage or with morpholine subunit (Fig. 1c).[3c] It has been shown that cytosine containing carbamate oligonucleotides form stable complexes with their complementary DNA, $(dG)_6$ and poly(dG), with *T*m values higher than their corresponding DNA–DNA duplexes.[4] Synthesis of di-T*T, tri-T*T*T, and tetrameric T*T*T*T, building blocks with internucleoside carbamate linkage 3′-NH-CO-*O*-5′ was achieved[5] by suitably protecting 5′-position and using these building blocks in the synthesis of chimeric oligonucleotides by standard phosphoramidite chemistry. Oligonucleotides containing one, two, or three carbamate units at 3′-end were found to have increased nuclease resistance but had no significant influence on the duplex thermal stability.[5] Chimeric carbamate-internucleoside dimer of 2′-deoxy-2′, 3′-secothymidine incorporated into the oligonucleotides showed less affinity towards complementary DNA as compared with the unmodified strand.[6]

Peptide nucleic acid, *aeg*PNA (Fig. 2a), a widely studied example of uncharged nucleic acid analogue based on polyamide backbone is a promising DNA mimic for antisense applications because of its superior DNA binding properties.[7] The main disadvantages of PNA as a viable drug candidate are its limited water solubility, inferior cellular uptake and poor discrimination of directional selective binding to the target DNA/RNA sequences.[1] Our efforts in this laboratory to address the shortcomings of PNA have led to the synthesis of chiral and/or positively charged water soluble pyrrolidine PNA analogues[8] derived from naturally occurring L-*trans*-4-hydroxy proline. The chiral 4-aminoprolylPNAs were designed by using all four diasteromers of 4-aminoproline to which the nucleobase was attached to the ring nitrogen via acetyl linker (Fig. 2b).[9] The homooligomers comprising all four diastereoisomers of 4-aminoprolyl peptide nucleic acids did not bind to the complementary DNA probably as a result of incompatible internucleobase distances arising from the introduction of backbone constraint. We envisaged that extending the backbone of 4-aminoprolylPNA by replacing the peptide linkage with a carbamate linkage (Fig. 2c) might release some of the geometric constraints in the *prolyl*PNA. The carbamate-linkage, though longer than the amide linkage, is shorter by 0.32 Å than a phosphodiester linkage[4] that may fulfill the requirement of internucleobase distance complementarity for target recognition and antisense activity. This manuscript reports the synthesis (2*S*, 4*S*)-4-amino-2-hydroxymethyl

*Corresponding author. Tel.: +91-20-5893153; fax: +91-20-5893153; e-mail: vakumar@dalton.ncl.res.in

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pyrrolidine carrying nucelobase thymine or cytosine to N1 via acetyl linker, **7** and **9** respectively. The preparation of carbamate-linked dimers, homooligomers and oligomers with mixed amide/carbamate backbone and their binding properties with complementary DNA using UV melting studies is also reported. A preliminary report of this work was presented as a poster at XIV IRT on nucleosides, nucleotides and nucleic acids.[10]

Results and Discussion

Synthesis of activated protected monomers 8, 10 and dimer 13

The (2*S*,4*S*)-4-Bocamino prolinol **5** was synthesized from N,O-protected (2*S*,4*R*)-4-hydroxyproline **1** as shown in Scheme 1. The 4*R*- hydroxy function in **1** was converted to the 4*R*-*O*-mesyl derivative **2** by treatment with mesyl chloride in dry pyridine. The 4*R*-*O*-mesyl derivative **2** on reacting with excess NaN_3 in DMF gave the 4*S*-azido compound **3**, accompanied by inversion at C4. Catalytic hydrogenation using Raney Ni at 40 psi readily converted this azido group to 4*S*-amino group with retention of N1-benzyloxycarbonyl group. The resulting 4*S*-amino compound without isolation was directly treated with *t*-Boc azide in DMSO/TEA to give the 4*S*-*t*-butoxycarbonylamino compound **4.** This, on reduction with $LiBH_4$ in THF afforded the protected prolinol derivative **5** which was then subjected to hydrogenation using Pd-C/H_2 at 60 psi to achieve N-1 deprotection. This was followed by coupling of pyrrolidine ring nitrogen with thyminyl acetic acid **6** using DCC/HOBt coupling conditions to get the thymine monomer **7**. Activation of primary alcohol in **7** was effected using *para*-nitrophenyl chloroformate in dioxane containing one equivalent of pyridine as base to yield the desired monocarbonate **10.** The activated monomer **10** was reasonably stable when stored at 4 °C under argon atmosphere.

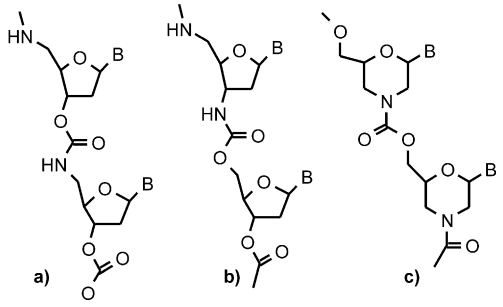


Figure 1. Carbamate linked nucleic acid analogues.

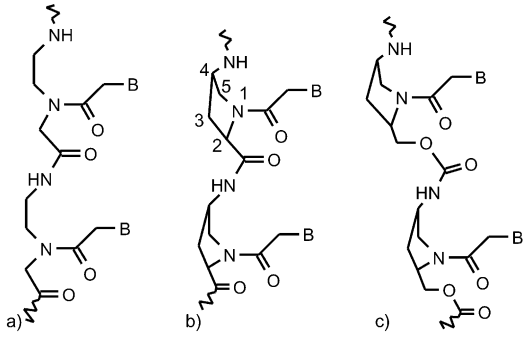
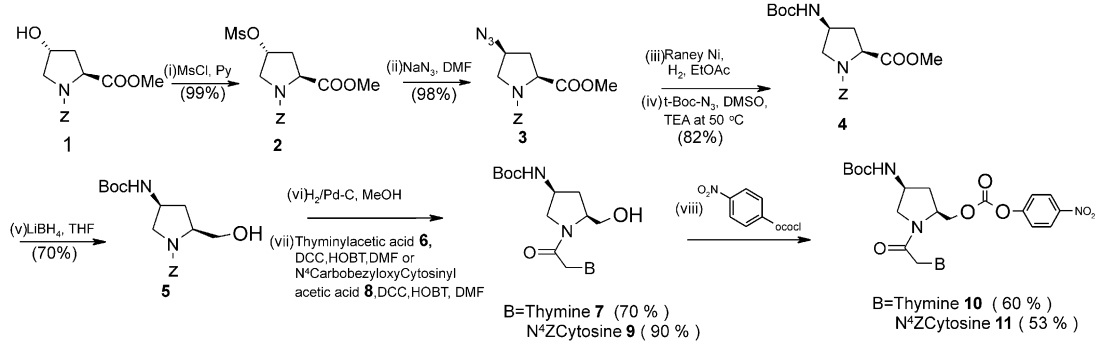


Figure 2. (a) aegPNA (b) aminoprolyl PNA (c) pyrrolidine carbamate PNA.

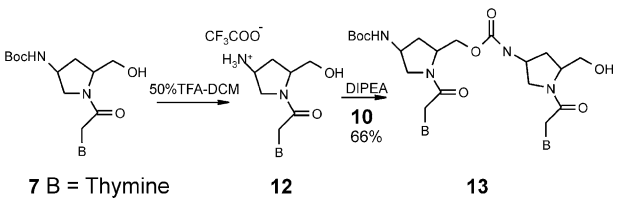
The common precursor **5**, after N1-deprotection using Pd/C in MeOH under H_2 pressure followed by coupling with N^4-benzyloxycarbonylcytosinyl acetic acid **8** in the presence of DCC/HOBt afforded **9.** (Scheme 1). Activation of the alcohol in compound **9** was effected in a manner similar to that described for the synthesis of thymine monomer **10** using Et_3N as base to obtain the *p*-nitrophenylcarbonate **11.** Carbamate linked pyrrolidinyl dimer **13** was synthesized from the thymine monomer **7** (Scheme 2) by first deprotection of *t*-Butyloxycarbonyl by treatment with 50%TFA/DCM to obtain the trifluoroacetate salt **12**. The salt **12**, after neutralization with DIPEA was coupled with one equivalent of the activated monomer **10** to furnish the dimer **13**. No undesirable carbonate product arising from the reaction of hydroxyl in the amino-alcohol **7** with the activated alcohol **10** was isolated. All the new compounds were characterized using ^1H, ^{13}C NMR and mass sprectrometry.

Solid phase synthesis of carbamate nucleic acids

The solid phase synthesis of carbamate-linked nucleic acids was carried out on Merrifield resin (1% cross-linked) by functionalizing it with *N*-*t*-Bocβ-alanine (0.68 mmol/g) followed by removal of *t*-Boc group with 50% TFA/DCM and neutralization with 5% DIPEA/DCM.



Scheme 1.



Scheme 2. Solid phase synthesis of carbamate nucleic acids.

The resin was partially capped using calculated amount of acetic anhydride in pyridine to decrease the loading value to approximately 0.3 mmol/g. The resin was reacted with three equivalents of activated thymine monomer **10** in the presence of catalytic amount of DIPEA for 2 h to give the thymine monomer bound to resin via carbamate linkage. The deprotection-coupling cycle was repeated twice to get the trimer and the reactions were monitored by Kaiser's test (Fig. 3).[11]

It is important to verify the stability of the carbamate linkage towards the conditions used to cleave the oligomer from the resin. In the case of solid phase synthesis of morpholine based carbamate oligonucleotides on 1% cross-linked aminomethyl polystyrene resin, the base deprotection as well as oligomer cleavage from the resin was effected with DBU/diethyl malonate in DMF to recover carbamate-linked hexamer in 95% yield.[12] Cyclic oligocarbamates synthesized on ester linked 2-chlorotrityl chloride resin were cleaved with hexafluoro-2-propanol (HFIP) without affecting the carbamate link.[13] In the present work, the resin-bound trimer **14** (H-t t t-β-alanine-MF) was subjected to acidic cleavage with trifluoromethanesulphonic acid (TFMSA) in trifluoroacetic acid (TFA) to cleave the benzyl ester and yield the carbamate oligomer having free carboxy-terminus. Alternatively, the oligomer bound-resin can be treated with methanolic ammonia at 55 °C for 16 h to yield peptide-C-terminal amide, followed by TFA/DCM (1:1) treatment for the removal N-terminal-*t*-Boc group. RP-18HPLC profile of the acidolysis product showed a major peak with a retention time of 21.3 min while in the case of ammonolysis product, there was only a minor peak (5%) at 21.4 min and a major peak at 5.9 min. MALDI-TOF mass spectrum of the crude product (21.3 min) from TFMSA/TFA cleavage reaction showed peaks at 1015.3 $(M+H)^+$, 1037.3 $(M+Na)^+$, 1057.5 $(M+K)^+$ [Calcd. for $C_{42}H_{55}N_{13}O_{17}=1013.38$] corresponding to the desired product. These results indicate that the carbamate linkage is stable to strong acidic conditions but not towards basic conditions. Various carbamate PNA oligomers synthesized in the present study are shown in Table 1. The octamer **CNA4** (H-t_8-β-ala) and the mixed base sequence **CNA5** are homooligomers with stereoregular backbone with all carbamate linkages.

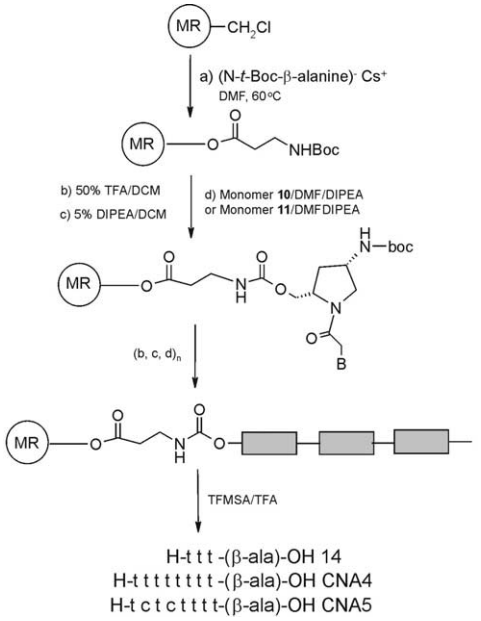


Figure 3. Schematic representation of solid phase synthesis of carbamate nucleic acids.

To study the compatibility of this modified unit in *aeg*PNAbackbone, the monomer unit **10** was introduced at predetermined positions in *aeg*PNA octamers. **CNA1** carries the modification at penultimate position of C-terminus, while **CNA2** and **CNA3** have modification in the middle and N-terminal respectively. The unmodified ***aeg*PNA** oligomer H-T_8-β-ala was used as control PNA for comparison and **DNA1** and **DNA2** are complementary to PNA sequences needed for duplex formation. The polypurine **DNA1** has GC clamps on both sides to prevent sliding of PNA. The cleavage of the assembled carbamate/peptide mixed as well as carbamate-linked homooligomers was carried out using TFMSA/TFA to yield sequences bearing free *C*-termini of the β-alanine. Under these conditions, the benzyloxycarbonyl group was also deprotected and no additional treatment was needed to remove N^4-Z-protection on the cytosine in **CNA5**. After the cleavage reaction, the oligomers were precipitated from methanol with dry diethylether. During precipitation, it was observed that the homooligomers **CNA4** and **CNA5** had considerable solubility in diethylether. They had poor water-solubility and hence gel-filtration on Sephadex G25, which requires aqueous conditions, could not be performed. LH-20 was used for their purification using isopropanol as eluant. All oligomers were further purified by reverse phase HPLC on a semi-preparative RP-C4 column. The purity of the oligomers was rechecked by reverse phase HPLC on an RP-18 column and their structural

Table 1. PNA/*py* CNA and DNA sequences used in the present study

Entry	Sequence
aeg PNA	H-TTTTTTTT-(β-ala)-OH
CNA1	H-TTTTTTtT-(β-ala)-OH
CNA2	H-TTTtTTTT-(β-ala)-OH
CNA3	H-tTTTTTTT-(β-ala)-OH
CNA4	H-t t t t t t t t-(β-ala)-OH
CNA5	H-t c t c t t t t-(β-ala)-OH
DNA1	5′-G C A A A A A A A A C G-3′
DNA2	5′-A A A A G A G A-3′

T denotes *aeg* monomer, t and c denote pyrrolidinyl monomer with thymine and cytosine nucleobase respectively.

integrity was confirmed by MALDI-TOF mass spectral analysis.[14]

Biophysical studies of CNA–DNA complexes

UV-Tm studies of the triplexes. UV-melting experiments were carried out with 2:1 stoichiometry for *aeg*PNA/CNA:DNA in 10 mM phosphate buffer at pH 7.3. Figure 4 shows the plot of % hyperchromicity at 260 nm versus the temperature and the Tm data derived from the first derivative plot are listed in Table 2. The control unmodified triplex (*aeg*PNA $T_8)_2$:DNA1 shows a Tm of 44.5 °C. The complex of CNA1 where the modified residue 't' is present in the penultimate position from the C-terminus of the *aeg*PNA sequence, had a lower Tm of 31.5 °C, while Tm of $(CNA3)_2$–DNA1 triplex with t at the N-terminus was 44 °C. In contrast, the stereoregular carbamate-linked oligomers CNA4 and CNA5 complexes with respective complementay DNA1 and DNA2 did not show a melting-transition and the hyperchromicity chages observed for these complexes were very low: $(CNA4)_2$:DNA1(7.1%) and $(CNA5)_2$:DNA2(9.3%) as compared to that for the mixmer *aeg*PNA-CNA–DNA complexes (18%–22%) which was even higher than that of *aeg*PNA:DNA triplexes (13%).

These results show that a single carbamate-linked 2*S*, 4*S* pyrrolidine modification towards the C-terminus (the penultimate residue) of the *aeg*PNA i.e., CNA1, causes a larger destabilization (ΔTm = −13 °C) as compared to an equivalent N-terminal modification (CNA3). The latter does not seem to affect the stability of the triplex significantly (ΔTm = −0.5 °C). At the same time a single modification in the center (CNA2) results in no complex formation with its complementary DNA1. Homoligomer pyrimidine sequences CNA4 and CNA5 with uniform carbamate linkages do not show sigmoidal transitions in UV melting experiments.

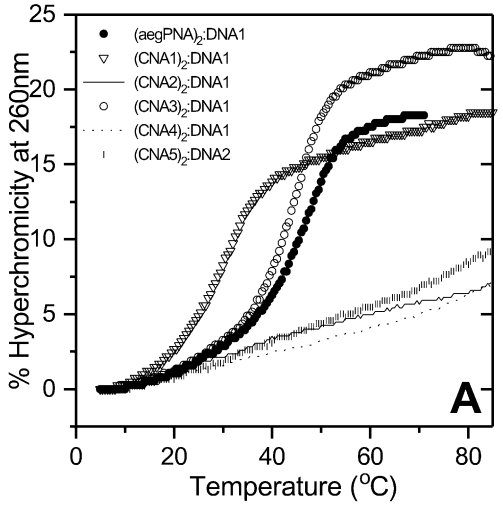


Figure 4. UV-Tm profiles of *aeg*PNA/CAN:DNA complexes.

Table 2. UV-Tm of aegPNA/CNA:DNA complexes[a]

Complex	Tm °C (ΔTm°C)	%Hyperchromicity
(*aeg*PNA)$_2$–DNA1	44.5	13
(CNA1)$_2$–DNA1	31.5 (−13)	18
(CNA2)$_2$–DNA1	nt	6
(CNA3)$_2$–DNA1	44 (−0.5)	22
(CNA4)$_2$–DNA1	nt	7
(CNA5)$_2$–DNA1	nt	9

[a]Experiments are repeated at least three times and the Tm values are obtained from the peaks of the first derivative plots of A_{260} against temperature.

Conclusions

An efficient solid phase synthesis of pyrrolidine carbamate nucleic acids has been developed. The pyrrolidinyl carbamate nucleic acids with (2*S*, 4*S*) strereochemistry having nucleobases thymine and cytosine were successfully incorporated in *aeg*PNA. In addition, synthesis procedure is developed and standardized to prepare oligomers with all carbamate linkages. The carbamate homoligomers were found to have poor water solubility. The UV-*T*m studies show that the (2*S*, 4*S*) pyrrolidinyl carbamate-linked homooligomers and with a single modification in the center of *aeg*PNA did not bind to DNA. Though the placement of carbamate linkage at C-terminus *aeg*PNA resulted in the destabilization of triplexes, the modification at the N-terminus had no effect on the stability of the complex. Interestingly, the percent hyperchromicity found in $(CNA1)_2$–DNA1 and $(CNA3)_2$–DNA1 triplexes was more than that for *aeg*PNA_2–DNA complexes indicating a better base-stacking in the case of CNA–DNA complexes.

These results indicate that in addition to the inter-nucleobase distance, other structural properties like sterically allowed constriction of the oligomers are important as well. The desired geometry could be controlled by the right kind of the stereochemistry in the pyrrolidinyl ring. In this regard, the carbamate-linkage needs to be explored more with the other three strereocisomers of the pyrrolidine unit.

Experimental

All solvents used were purified according to the standard procedures. The reactions were monitored by TLC on silica gel. Usual work up implies sequential washing of the organic extract with water and brine followed by drying over anhydrous sodium sulphate and evaporation under vacuum. Column chromatography was performed for purification of compounds on 100–200 mesh silica gel, except in case of activated monomers in which case purification was done by flash column using 200–400 mesh silica gel. CNA/PNA purifications were performed on an RPC-C4 semi-preparative column attached to HPLC system equipped with Jasco-UV970 and purification was carried out using 0.1%TFA using gradient CH_3CN:H_2O buffer. The purity of oligomers was further assessed by RP-18 analytical HPLC column (25×0.2 cm, 5 μm) using gradient method: A→50%B in 30 min, where buffer A = 0.1%TFA/H_2O and buffer B = 0.1% TFA in CH_3CN:H_2O 1:1 with flow rate 1 mL/min and the eluant was monitored at 260 nm.

Methyl (2*S*, 4*R*)-1-benzyloxycarbonyl-4-methanesulfonyloxypyrrolidine-2-carboxylate (2). Compound **1** (9.7 g, 34.6 mmol) was taken in dry pyridine (75 mL) and mesyl chloride (3.2 mL, 41.5 mmol) was added dropwise over a period of 30 min while maintaining the temperature of the flask at 0–5 °C. After stirring for 1 h, the reaction mixture was kept in refrigerator overnight. Pyridine was removed under vacuum in a rotavapor and traces of pyridine were removed by coevaporation with toluene (2×20 mL). EtOAc (200 mL) was added to the residue and washed with water. The aqueous layer was further extracted with EtOAc (3×50 mL) and the organic layers were pooled, washed sequentially with water, brine and dried over anhydrous Na_2SO_4. Upon removal of EtOAc, 4*R*-mesyl-N1-benzyloxycarbonyl-proline methyl ester **2** was obtained (12.3 g, Yield = 99%, R_f = 0.4, EtOAc:Petroleum ether 6:4, $\alpha_D^{27}(CHCl_3) = -45.6$).

^{1}H NMR ($CDCl_3$) δ 7.35–7.25 (2s 5H), 5.3–5.0 (2m 3H), 4.5 (dd 1H), 4.0–3.8 (m 2H), 3.75 & 3.5 (2s 3H), 3.0 (2s 3H), 2.7–2.2 (m 2H).

^{13}C NMR ($CDCl_3$) (rotamers) δ 172.06, 154.38, 153.90, 136.07, 129.53, 129.13, 128.94, 79.06, 78.69, 68.51, 58.22, 53.77, 53.58, 53. 44, 53.36, 39.65, 38.44, 37.30.

ESI-MS (MeOH + water + ammonium acetate) 358.05 $(M+H)^+$, 375.05 $(M+NH_4)^+$ (calcd for $C_{15}H_{19}NSO_7$: 357).

Methyl(2*S*, 4*S*)-4-azido-1-benzyloxycarbonylpyrrolidine-2-carboxylate (3). The 4*S* mesyl derivative **2** (12.3g 34.3mmol) was taken in dry DMF (100 mL) and NaN_3 (17.8 g, 0.27 mol) was added into it. The reaction mixture stirred at 70 °C for 7–8 h. DMF was removed under vacuum and the residue was taken in EtOAc (200 mL) and washed with water. The aqueous layer was washed with EtOAc (3×50 mL) and all organic layers were pooled together and washed with water, brine and dried over anhydrous Na_2SO_4. The solvent was removed to obtain the azido compound **3** (10.27 g, yield = 98.5%, R_f = 0.36, EtOAc:Petroleum ether 4:6, α_D^{27} $(CHCl_3) = -33.3$).

^{1}H NMR ($CDCl_3$) δ 7.27–7.23 (m 1H), 5.17–4.90 (m 2H), 4.43–4.31 (dd J = 8, 16, 1H), 4.16–4.04 (bm 1H), 3.70–3.45 (m 5H), 2.33–2.17 (m 1H), 2.15–2.02 (m 1H).

^{13}C NMR ($CDCl_3$) (rotamers) δ 170.84, 170.82, 153.38, 153.02, 135.74, 127.4, 126.9, 126.7, 65.97, 58.2, 57.3, 56.93, 56.6, 51.09, 50.5, 50.1, 34.8, 33.85.

IR (neat) cm^{-1} 2953, 2106, 1753, 1711, 1499, 1416.

ESI-MS (MeOH + water + ammonium acetate) 305.05 $(M+H)^+$, 322.05 $(M+NH_4)^+$ (calcd for $C_{14}H_{16}N_4O_4$: 304)

Methyl (2*S*, 4*S*)-1-benzyloxycarbonyl-4-*tert*-butoxycarbonylamino pyrrolidine-2-carboxylate (4). The azide **3** (3.11 g, 10.2 mmol) was dissolved in methanol (1.5 mL) and treated with suspension of Raney Nickel (4 mL) in water. The mixture was subjected to reduction under hydrogen pressure (40 psi) for 1.5 h in a Parr hydrogen apparatus. Raney Ni was filtered through Celite and washed with methanol, which was then evaporated under vacuum to yield a pale yellow solid. This was dissolved in DMSO (20 mL) and treated with *t*-Boc-N_3 (1.7 mL, 12 mmol) followed by addition of Et_3N (1.8 mL, 13 mmol). After stirring the reaction mixture for 8 h, the contents were poured into a beaker containing ice-cold water (100 mL), that led to the formation of a white precipitate. The precipitate was extracted in EtOAc and sequentially washed with water, aqueous $KHSO_4$ (10%, w/v), water and brine. Upon evaporation of ethyl acetate layer, compound **4** was obtained (3.17 g, yield = 82%, R_f = 0.33, EtOAc: Petroleum ether 1:1, $\alpha_D^{27}(CHCl_3) = -15.5$).

^{1}H NMR ($CDCl_3$) δ 7.32–7.28 (m 5H), 5.20–4.96 (m 2H), 4.86–4.64 (bm 1H), 4.46–4.34 (m 1H), 4.33–4.16 (bm 1H), 3.86–3.76 (m 1H), 3.71–3.54 (2s 3H), 3.43–3.27 (m 1H), 2.28–2.09 (m 2H), 1.40 & 1.38 (2s 9H).

^{13}C NMR ($CDCl_3$) (rotamers) δ 172.4, 172.3, 155.0, 154.0, 136.1, 128.3, 127.9, 127.7, 79.8, 67.1, 57.7, 57.5, 52.2, 52.0, 51.7, 49.5, 48.9, 36.7, 35.6, 28.2.

ESI-MS (MeOH + water + ammonium acetate) 379.05 $(M+H)^+$, 396.05 $(M+NH_4)^+$ (calcd for $C_{19}H_{26}N_2O_6$: 378)

(2*S*, 4*S*)-1-benzyloxycarbonyl-4-tert-butoxycarbonylamino-pyrrolidine-2-methanol 5. The ester **4** (1 g, 2.6 mmol) was coevaporated with dry THF (2×10 mL) and then dissolved in dry THF (20 mL). The solution was cooled to 0 °C and $LiBH_4$ (0.1 g, 5.3 mmol) was added in two portions. After stirring it for 3.5 h, the reaction was quenched with saturated aqueous NH_4Cl solution. The solvent was removed under vacuum, the residue was dissolved in EtOAc and the resulting solution was washed with water and brine. EtOAc layer was dried over anhydrous Na_2SO_4, solvent was evaporated and the residue (0.83 g) was purified by column chromatography to afford **5** (0.65 g, yield = 70%, R_f = 0.25, EtOAc: Petroleum ether 1:1, $\alpha_D^{27}(CHCl_3) = -22.6$).

^{1}H NMR ($CDCl_3$) δ 7.45–7.3 (s 5H), 5.5 (bs 1H), 5.1 (s 2H), 4.3–4.15 (m 1H), 4.1–3.9 9m 2H), 3.4 (bs 1H), 3.4–3.2 (m, 1H), 2.5–2.3 (m, 1H), 1.7–1.5 (m, 1H), 1.4 (s, 9H).

^{13}C NMR ($CDCl_3$) (rotamers) δ 155.9, 136.7, 128.9, 128.35, 128.28, 79.7, 67.5, 64.6, 59.58, 54.28, 49.17, 49.03, 34.8, 28.77.

ESI-MS (MeOH + water + ammonium acetate) 351.05 $(M+H)^+$, 368.05 $(M+NH_4)^+$ (calcd for $C_{18}H_{26}N_2O_5$: 350)

(2*S*, 4*S*)-4-t-butoxycarbonylamino-1- [(thymin-1-yl)methylcarbonyl]-pyrrolidin-2-methanol (7). The alcohol **5** (3 g, 8.57 mmol) was dissolved in methanol and 10%Pd-C (0.3 g) was added into it. This slurry was subjected to hydrogenation at 60 psi H_2 pressure for 7 h. The catalyst

was filtered off over Celite and filtrate was evaporated under vacuum to obtain the amine as yellow solid (~1.9 g). The free amine was taken in dry DMF (30 mL) to which thyminyl acetic acid **6** (1.73 g, 9.4 mmol) and HOBt (0.578 g, 4.28 mmol) were added. After cooling the mixture in an ice bath, DCC (1.94 g 9.4 mmol) was added and stirred for 4 h. The DCU that precipitated was removed by filtration through Celite and the solvent was removed under vacuum followed by purification of the residue by silica gel column to give **7** (2.78 g, yield = 85%, R_f = 0.3, MeOH:DCM 1:9, $\alpha_D^{27} = -67.5$, mp = 58–60 °C).

^{1}H NMR ($CDCl_3 + D_2O$) δ 10.2 (bs, 0.4H), 7.0 (s, 1H), 4.9–4.5 (m, 1H), 4.4–4.0 (m, 4H), 3.6–3.1 (m, 1H), 1.9 (s, 3H), 3.1–2.8 (m, 2H), 2.65–2.35 (m, 2H), 1.5 (2s, 9H).

^{13}C NMR (CD_3OD) δ 168.2, 167.9, 166.8, 157.6, 153.1, 143.5, 143.1, 111.2, 80.3, 65.2, 62.8, 60.4, 59.5, 53.6, 35.6, 33.8, 28.6, 12.1.

ESI-MS (MeOH + water + ammonium acetate) 383.05 ($M + H^+$), 400.05 ($M^+ + NH_4$) (calcd for $C_{17}H_{26}N_4O_6$: 382)

{(2*S*, 4*S*))]-4-*tert*-butoxycarbonylamino-1-[(thymin-1-yl)-methylcarbonyl]pyrrolidin-2-yl}methyl 4-nitrophenyl carbonate (10). *tert*-Boc-protected thymine monomer **7** (0.53 g, 1.38 mmol) was dried by co-evaporation with dry dioxane (3×5 mL) under reduced pressure. The resulting solid was dissolved in dioxane:Py mixture (11 mL; 10:1) and cooled to 10 °C. *p*-nitrophenyl chloroformate (0.56 g, 2.78 mmol) was added to the reaction vessel in portions under anhydrous conditions and mixture was stirred for 4 h after second addition. The solvent was evaporated, without heating, under reduced pressure to obtain a crude product (1.6 g), which was purified by flash column chromatography using ethyl acetate/petroleum ether (7:3) to obtain the pure product **10** (0.46 g, yield = 60%, R_f = 0.34, EtOAc:Petroleum ether 7:3, $\alpha_D^{27} = -71$, mp = 80 °C).

^{1}H NMR ($CDCl_3$) δ 8.25 (d, 2H, J = 7 Hz), 7.4 (d, 2H J = 7 Hz), 7.0 (s 1H), 5.2 (bs, 1H), 4.8–4.1 (m, 7H), 4.05–3.7 (m 1H), 2.6–2.2 (m 1H), 2.15–1.95 (m 1H), 1.9 (s 3H), 1.4 (2s, 9H).

^{13}C NMR ($CDCl_3$) (rotamers) δ 165.9, 164.17,155.5, 155.3, 152.1, 151.1, 151.3, 145.5, 140.85, 126.09, 125.3, 121.7, 115.6, 110.8, 80.3, 68.3, 55.66, 52.8, 49. 69, 49.05, 32.95, 28.36, 12.22.

ESI-MS (MeOH + water + ammonium acetate) 547.01 (M^+), 564.01 $(M + NH_3)^+$ (calcd for $C_{24}H_{29}N_5O_{10}$: 547)

(2*S*, 4*S*))-1-[(cytosin-1-yl)methylcarbonyl]-4-*tert*-butoxycarbonylaminopyrrolidin-2-methanol (9). The Z group in *t*-Boc protected 4-amino alcohol **5** (1.62 g, 4.63 mmol) was deprotected to obtain the free amine (1 g). To this amine (1 g, 4.6 mmol) in dry DMF (30 mL), N^4-cbz protected N1-cytosine acetic acid **8** (1.54 g, 5.09 mmol) and HOBt (0.3 g, 2.3 mmol) were added and cooled in an ice bath. DCC (1.1 g, 5.1 mmol) was added to this mixture and the reaction was stirred at room temperature for 6 h. It was filtered over Celite and the filtrate was evaporated to dryness under reduced pressure. The residue was purified by silica gel column chromatography using MeOH/DCM to afford pure **9** (1.85 g, yield = 80%, R_f = 0.2, MeOH:DCM 3:97, $\alpha_D^{27} = -54$, mp = 70 °C).

^{1}H NMR ($CDCl_3$) δ 7.6 (d, 1H), 7.4–7.1 (s & d 6H), 5.2 (s 2H), 4.5–3.35 (m, 7H), 2.5–2.25 (m, 1H), 1.95–1.65 (m, 1H), 1.4 (s, 9H).

^{13}C NMR ($CDCl_3$) δ 165.7, 163.1, 155.8, 155.5, 152.7, 149.8, 135.1, 128.3, 128.1, 127.8, 95.1, 79.3, 77.1, 67.3, 63.5, 62.6, 59.5, 53.0, 51.2, 35.5, 33.6, 28.1.

FAB-MS 502 $(M + H)^+$, 524 $(M + Na)^+$ (calcd for $C_{24}H_{31}N_5O_7$: 501.2).

{(2*S*, 4*S*)-1-[(cytosin-1-yl)methylcarbonyl]-4-*tert*-butoxycarbonylaminopyrrolidin-2-yl}methyl 4-nitrophenyl carbonate (11). The cytosine monomer **9** (0.5 g, 1 mmol) was coevaporated with dry dioxane (2×10 mL) and then treated with *p*-nitrophenyl chloroformate (0.6 g, 3 mmol) and Et_3N (2.7 mL, 20 mmol) in dry dioxane (10 mL). After stirring it overnight, solvent was evaporated under vacuum and residue was taken in EtOAc and subsequently washed with aqueous NaOH (0.01N, 2×5 mL), water and brine. The organic layer was dried over anhydrous. Na_2SO_4 and concentrated to get **11** (340 mg, yield = 53%, R_f = 0.5, EtOAc, $\alpha_D^{27} = -68$, mp = 94 °C).

^{1}H NMR ($CDCl_3$) δ 8.25 (d 2H, J = 7.0 Hz), 7.1 (d 1H J = 7.3), 7.5–7.1 (d, s & d, 7H), 5.4 (bs, 1H), 5.25 (s, 2H), 4.8–3.8 (m, 7H), 2.2–1.6 9, m, 2H), 1.4 (s, 9H).

FAB-MS 667$(M + H)^+$, 689 $(M + Na)^+$ (calcd for $C_{31}H_{34}N_6O_{11}$: 666.2)

Carbamate dimer (13). The thymine monomer **7** (100 mg, 0.26 mmol) was treated with 50%TFA/DCM (2 mL) and stirred at room temperature for 30 min. The solvent and acid were removed under reduced pressure and coevaporated twice with dry dichloromethane. To this amine salt **12**, DIPEA (89 μL, 0.52 mmol), activated thymine monomer **10** (142 mg, 0.26 mmol) and dry DMF (5 mL) were added. After stirring it for 3 h at room temperature, solvent was removed under vacuum and the residue was purified by column to afford the dimer **13** (116 mg, yield = 65%, R_f = 0.3, MeOH:EtOAc 23:77).

FAB-MS. 691 $(M + H)^+$, 713 $(M + Na)^+$, (calcd for $C_{30}H_{42}N_8O_{11}$: 690.29).

UV melting experiments

The melting experiments were carried out in 10 mM phosphate buffer at pH 7.3. *aeg*PNA/*py*CNA oligomers and their complementary DNA oligonucleotide pairs were taken at concentrations of 2.5 μM and 1.25 μM respectively. In the case of **CNA4** and **CNA5**, the stock

solutions of the oligomers were prepared in DMSO and then 10–15 μL of the solution was diluted with phosphate buffer to make up the required concentration and volume. The triplexes were formed by an annealing the appropriate samples by heating at 85 °C for 2 min followed by slow cooling to room temperature for ~30 min and then refrigerated overnight. Melting experiments were carried out at a heating rate of 0.5 °C/min, while the absorbance at 260 nm was monitored at every temperature. The Tm values were obtained from the first derivative curves of the A_{260} vs temperature plots.

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References and Notes

1. Uhlmann, E.; Peyman, A.; Breiphol, G.; Will, D. W. *Angew. Chem., Int. Ed. Engl.* **1998**, *37*, 2796.
2. Mungall, W. S.; Kaiser, J. K. *J. Org. Chem.* **1977**, *42*, 703.
3. (a) Gait, M. J.; Jones, A.Stanley; Walker, R. T. *J. Chem Soc. Perkin.* **1974**, *1*, 1684. Coull, J. M.; Carlson, D. V.; Weith, H. L. *Tetrahedron Lett.* **1987**, *28*, 745. (b) Kristina, M. K.; Just, George *Bioorg. Med. Chem. Lett.* **1994**, *4*, 435. (c) Stirchak, E. S. P.; Summerton, J. E.; Weller, D. D. *Nucleic Acids Res.* **1989**, *17*, 6129.
4. Stirchak, E. S. P.; Summerton, J. E.; Weller, D. D. *J. Org. Chem.* **1987**, *52*, 4202.
5. Habus, I.; Temsamani, J.; Agrawal, S. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1065.
6. Habus, I.; Agrawal, S. *Nucleosides and Nucleotides* **1995**, *14*, 1853.
7. Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O. *Science* **1991**, *254*, 1497.
8. Kumar, V. A. *Eur. J. Org. Chem* **2002**, 1021.
9. Gangamani, B. P.; Kumar, V. A.; Ganesh, K. N. *Tetrahedron* **1996**, *52*, 15017. Gangamani, B. P.; Kumar, V. A.; Ganesh, K. N. *Tetrahedron* **1999**, *55*, 177.
10. Meena; Kumar, V. A.; Ganesh, K. N. *Nucleosides and Nucleotides and Nucleic Acids* **2001**, *20*, 1193.
11. Kaiser, E.; Bossinger, C. D.; Colescott, R. L.; Olsen, D. B. *Anal. Chim. Acta* **1980**, *118*, 149.
12. Wang, H.; Weller, D. D. *Tetrahedron Lett.* **1991**, *32*, 7385.
13. Warrass, R.; Wiesmuller, K.-H.; Jung, G. *Tetrahedron Lett.* **1998**, *39*, 2715.
14. CNA1 (MALDI-TOF M + H 2260.5 Calculated 2259.8), CNA2 (MALDI-TOF M + H 2260.8 Calculated 2259.8), CNA3 (MALDI-TOF M + H 2260.5 Calculated 2259.8), CNA5(MALDI-TOF M + Na 2546.4 Calculated 2523.9)