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Synthesis and Hybridization Properties of DNA–PNA Chimeras Carrying 5-Bromouracil and 5-Methylcytosine

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Abstract—The preparation of 5-bromouracil and 5-methylcytosine peptide nucleic acid (PNA) monomers is described. These PNA monomers were used for the preparation of several DNA–PNA chimeras and their hybridization properties are described. The substitution of cytosine by 5-methylcytosine in DNA–PNA chimeras increased duplex stability while substitution of thymine by 5-bromouracil maintained it. Moreover, binding of DNA–PNA chimeras to double-stranded DNA to form triple helices was studied. In contrast to DNA, the presence of 5-methylcytosine and 5-bromouracil in DNA–PNA chimeras destabilized triple helix.

Introduction

Peptide nucleic acids (PNA) are oligonucleotide analogues in which the sugar-phosphate backbone has been replaced by *N*-(2-aminoethyl)glycine units.[1] In spite of the change in the backbone structure, PNA molecules bind strongly to complementary DNA and RNA sequences,[2] and they are achiral and uncharged. For these reasons PNA oligomers have a strong potential as therapeutic agents, diagnostic tools and probes in molecular biology.[3]

Although PNA oligomers have excellent binding properties, they show poor solubility and a tendency to self-aggregation.[4] Some of these problems have been solved by preparing DNA–PNA chimeras. These chimeras are more water soluble, maintain the ability of DNA to activate RNase H and retain some of the enhanced binding and nuclease resistance properties of PNA.[5–12]

The preparation of DNA–PNA chimeras has been described by several authors.[5–12] The most common strategy is to use the MMT group for the temporary protection of the backbone amino function, and acyl groups for the exocyclic amino functions of the nucleobases.[5–8] The MMT is removed under mild conditions (3% TCA in DCM), and the nucleobase-protecting groups are removed using concentrated aqueous ammonia. These conditions are similar to those used in DNA synthesis allowing the preparation of PNA–DNA chimeras.[5–12]

We report the preparation of DNA–PNA chimeras carrying 5-bromouracil and 5-methylcytosine. Substitution of cytosine by 5-methylcytosine in oligonucleotides increases the stability of both double[13,14] and triple[15,16] helices. Oligonucleotides carrying 5-bromouracil are used in photo-cross-linking experiments,[17] in X-ray diffraction studies,[18] as non-radioactive labels[19] and for triple-helix stabilization.[16,20] The effect of the incorporation of 5-bromouracil and 5-methylcytosine in PNA oligomers or DNA–PNA chimeras on these properties remains to be determined. A PNA oligomer carrying 5-bromouracil has been prepared[21] using Boc-chemistry, which is, however, incompatible with the synthesis of DNA–PNA chimeras.

Recently, a new strategy has been developed for the preparation of MMT-protected PNA monomers which

Keywords: 5-bromouracil; duplex stability; 5-methylcytosine; PNA; PNA–DNA chimeras.

Abbreviations: ACN: acetonitrile; AcOEt: ethyl acetate; AcOH: acetic acid; Boc: *tert*-butoxycarbonyl; DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene; DCM: dichloromethane; DIP: diisopropylcarbodiimide; DIPEA: diisopropylethylamine; DMAP: 4-*N*,*N*-(dimethylamino)pyridine; DMF: *N*,*N*-dimethylformamide; DMT: dimethoxytrityl; Et_3N: triethylamine; HATU: *N*-[(dimethylamino)-1*H*; 1,2,3-triazolo-[4,5-*b*]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HOOBt: 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; MMT: monomethoxytrityl; MeOH: methanol; PEG-PS: polyethyleneglycol-polystyrene; PNA: peptide nucleic acid; TCA: trichloroacetic acid; TFA: trifluoroacetic acid; THF: tetrahydrofuran; .

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uses the 2-cyanoethyl group for the protection of the carboxylic function.[22] This group is removed by β-elimination with a strong but non-nucleophillic base in conditions that are compatible with acid labile (DMT and Boc) protecting groups and acyl protecting groups.[22] In this paper we used this method to prepare MMT-protected PNA monomers of 5-bromouracil and 5-methylcytosine. These monomers were used for the synthesis of several DNA–PNA chimeras carrying 5-bromouracil and 5-methylcytosine. Base-pairing properties of the modified DNA–PNA chimeras are described.

Results and Discussion

Synthesis of the PNA monomers

The preparation of the PNA monomer of 5-bromouracil is illustrated in Figure 1. First, the 5-bromouracil acetic acid derivative (**1**) was prepared by direct alkylation of 5-bromouracil with bromoacetic acid as described for the thymine derivative.[23] Coupling of compound **1** to *N*-MMT-2-aminoethyl-glycine 2-cyanoethyl ester using DIP and HOOBt as condensing agents in the presence of *N*-ethylmorpholine[5] yielded the CE-protected PNA monomer (**2**). Finally, compound **2** was treated with DBU[22] to give the desired 5-bromouracil PNA monomer (**3**), which was isolated as triethylammonium salt in 58% yield.

The preparation of the PNA monomer of 5-methylcytosine required the conversion of thymine to 5-methylcytosine, the introduction of the carboxymethyl group, and the protection of the 4-amino function. The synthesis scheme is illustrated in Figure 2. First we introduced the carboxymethyl group in thymine. Alkylation of thymine was performed with *t*-butyl bromoacetate in the presence of DBU, yielding compound **4** in 81% yield. The *t*-butyl ester was selected because it is stable to ammonia, which was later used in the conversion of thymine to 5-methylcytosine. The next step was the activation of position 4 of thymine to introduce an amino group. Compound **4** was activated with mesitylenesulfonic chloride followed by displacement of the mesitylenesulfonyl group with *o*-nitrophenol. The *o*-nitrophenyl thymine derivative (**5**) was isolated in 64% yield. Displacement of the *o*-nitrophenyl group by ammonia yielded the desired carboxymethyl derivative of 5-methylcytosine (**6**) in 68% yield.

The introduction of the protecting group for the exocyclic 4-amino function of 5-methylcytosine was troublesome. First, the isobutyryl group was introduced by reaction of compound **6** with isobutyryl anhydride. The isobutyryl derivative was isolated in good yields but the isobutyryl group did not resist the TFA treatment needed for the elimination of the *t*-butyl group (data not shown). Then, the more stable benzoyl group was selected. Reaction of compound **6** with benzoic anhydride yielded the benzoyl derivative **7** in 65% yield, which was treated with TFA giving the desired protected derivative

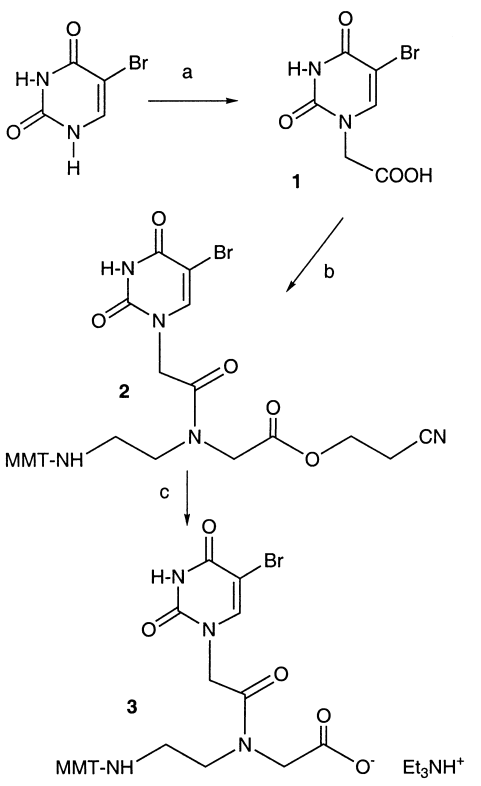

Figure 1. Preparation of the PNA monomer of 5-bromouracil. (a) bromoacetic acid, NaOH; (b) *N*-(2-(MMT-amino)ethyl)glycine 2-cyanoethyl ester, DIP, HOOBt, *N*-ethylmorpholine; (c) DBU.

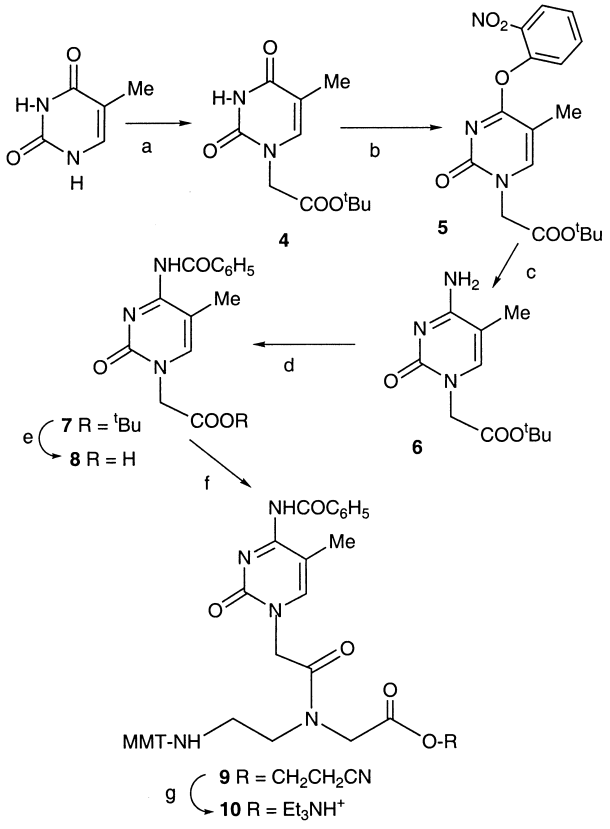

Figure 2. Preparation of the PNA monomer of 5-methylcytosine. (a) *t*-Butyl bromoacetate, DBU; (b) 2-mesitylenesulfonyl chloride, DMAP followed by *o*-nitrophenol, 1,4-diazabicyclo[2.2.2]octane; (c) ammonia; (d) benzoyl choride, pyridine; (e) TFA; (f) *N*-(2-(MMT-amino)ethyl)glycine 2-cyanoethyl ester, DIP, HOOBt, *N*-ethylmorpholine; (g) DBU.

of 5-methylcytosine (compound **8**). This derivative was reacted with the MMT, CE-protected *N*-(2-aminoethyl)glycine backbone to give compound **9**, which was converted to the desired 5-methylcytosine PNA monomer **10**.

Synthesis of DNA–PNA chimeras

Several DNA–PNA chimeric molecules carrying 5-bromouracil (B) and 5-methylcytosine (M) were prepared using solid-phase protocols. Polyethyleneglycol-polystyrene (PEG-PS) carrying an aminohexyl linker[5] was used as solid support. The sequences were: I 5′ CTTCC*TCCTCT*-CONH-hexyl-OH, II 5′ MTTCCM M*TCCTCT*-CONH-hexyl-OH, III 5′ CTTCC*TMMT M*-CONH-hexyl-OH, IV 5′ MTTMM*TMMTMT*-CONH-hexyl-OH and V 5′ CTTCC*TCCBCB*-CONH-hexyl-OH. The PNA part is written in italics. The last PNA monomer (*T*) is a T linker carrying a hydroxyl group protected with the MMT group to connect with the oligonucleotide part.[6,10] The addition of 5-methyl-C in the DNA part was performed using the commercially available phosphoramidite derivative in which the exocyclic amino group is protected with the benzoyl group. The PNA part was synthesized manually and the DNA part using an automatic DNA synthesizer as described previously.[22] Coupling efficiencies of the 5-bromouracil and 5-methylcytosine PNA monomers were similar to those of the natural bases.

After the assembly of the sequences, supports were treated with concentrated ammonium hydroxide. The ammonia deprotection of DNA–PNA chimera containing 5-bromouracil (V) was performed at room temperature to avoid the degradation of 5-bromouracil observed in oligonucleotides.[20] The rest of the chimeras were deprotected at 55 °C. The resulting products were purified by reversed-phase HPLC. In all cases a major peak was observed, which had the expected molecular weight as measured by electrospray mass spectrometry (see Fig. 3).

Hybridization properties of DNA–PNA chimeras

The influence of 5-bromouracil and 5-methylcytosine on duplex stability was evaluated in a polypurine-polypyrimidine duplex in which thymine had been replaced by 5-bromouracil and cytosine by 5-methylcytosine. Melting temperatures at medium salt concentration are shown in Table 1. The duplex constituted of DNA–PNA chimera I (without any base substitution) and its complementary DNA strand had a lower melting temperature than a pure DNA duplex (37 versus 42 °C). This decrease is in agreement with previously reported data[10,12] and is attributed to the unfavorable geometry of the DNA–PNA junction.[10] Substitution of thymine by 5-bromouracil in DNA–PNA chimeras had no influence on duplex stability (37 °C for chimera V versus 37 °C for chimera I). The same substitution in oligodeoxynucleotides led to a slight increase in duplex stability[24] but maintenance of duplex stability is enough for most of the applications described for 5-bromouracil.[17–19] When cytosine was substituted by 5-methylcytosine in DNA–PNA chimeras the duplex stability increased from 37 °C for chimera I (no substitution), to 39–40 °C for chimeras II and III (3 substitutions) and to 45 °C for chimera IV (6 substitutions). Introduction of a methyl group in the cytosine had a beneficial effect on duplex stability in both DNA and PNA parts. By analogy with DNA oligonucleotides[13,14] and hexitol nucleic acids[25] the stabilizing effect is most likely due to increased stacking interactions or dehydration of the helix. This increase is of special interest for the potential applications of PNA and DNA–PNA chimeras.[3,4]

The influence of 5-bromouracil and 5-methylcytosine in triple-helix formation was studied with the sequence described by Xodo et al.[15] Triple helices were formed by mixing a hairpin molecule (26-mer) with different all-pyrimidine DNA–PNA chimeras (11-mer). The UV absorbance at 270 nm was then followed as a function of temperature at pH 5.5 and 6.0 and at two different salt concentrations (0.1 and 1 M NaCl in 0.1 M sodium phosphate/citric acid buffer). As described earlier, two transitions were observed.[15] The first transition is due to dissociation of the DNA–PNA chimera from the triplex (triplex to duplex) and the second transition to the denaturation of hairpin (duplex to single strand). Table

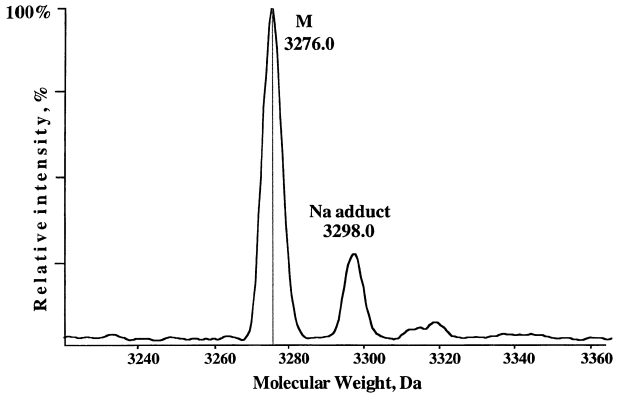

Figure 3. MS analysis (electrospray) of purified chimera V. The sample (PNA–DNA chimera V, 30 μg) was dissolved in 1 ml of 50% water/methanol mixture. One microliter aliquot was taken up and analyzed on a triple quadrupole mass spectrometer API III (PE Sciex, Ontario) equipped with nanoelectrospray ion source developed in EMBL. The figure presents an image deconvoluted using BioMultiview software (PE Sciex, Ontario) from the original spectrum containing the series of multiply charged ions.

Table 1. Melting temperatures (Tm) of the duplexes containing DNA–PNA chimeras (0.15 M NaCl, 0.05 M Tris–HCl pH 7.5). PNA part in italics. M = 5-methyl-C; B = 5-bromo-U and *T* = T-hydroxyethylglycine as PNA–DNA linker

Compound	Sequence (5′- > 3′, *N*- > *C*)	Tm (°C)
DNA	CTTCCTCCTCT[a]	42
Chimera I	CTTCCT*CCTCT*[a,b]	37
Chimera II	MTTMM*TCCTCT*[a,b]	40
Chimera III	CTTCC*TMMTMT*[a,b]	39
Chimera IV	MTTMM*TMMTMT*[a,b]	45
Chimera V	CTTCC*TCCBCB*[a,b]	37

[a]Complementary strand 5′ AGAGGAGGAAG 3′.
[b]Terminal 6-(hydroxyhexyl)carboxamide.

2 shows the melting temperatures for triple-helix dissociation. In order to simplify the table, the melting temperature of the duplex (around 75 °C) was omitted. Triplex dissociation of DNA–PNA chimeras was only observed at pH 5.5 and medium salt conditions (0.1 M NaCl), and melting temperatures were between 17 and 24 °C. In contrast, the all-DNA oligomer had a melting temperature for triplex dissociation of 38 °C at 0.1 M NaCl, and 43 °C at 1 M NaCl (both at pH 5.5) and no transition was observed for the all-PNA molecule either at 0.1 or 1 M NaCl, or at pH 5.5 or 6.0. Most of the triple helix stabilization properties of PNA are described for PNA•PNA•DNA triple helices.[3,4] PNA binding to duplex DNA to form PNA•DNA•DNA triple helices was described to be at least three orders of magnitude less efficient.[26] This is consistent with the absence of a triplex melting curve for the all-PNA oligomer. There is no previous data on the binding of DNA–PNA chimeras to duplex DNA. Our results indicate that DNA–PNA chimeras bind to duplex DNA to form triple helices at low pH and at medium salt concentration (0.1 M NaCl). These triple helices are less stable than all-DNA triple helices, but more stable than triple helices formed with PNA oligomers, which are not observed. Most probably the binding to duplex DNA is given by the DNA part of the chimeras but, on the other hand, triple helices formed by DNA–PNA chimeras are only observed at 0.1 M NaCl but not at 1 M NaCl. This is probably due to the presence of the PNA part, since pure DNA triplexes are stable at high concentrations of monovalent cations because salts reduce the repulsion of the phosphate groups of three strands.[27]

Substitution of thymine by 5-bromouracil and cytosine by 5-methylcytosine in DNA–PNA chimeras induced a significant decrease in the stability of triplex indicated by lowering the melting temperatures (17–21 °C for chimeras II–IV versus 24 °C for chimera I). In contrast, the same substitutions are reported to induce a strong stabilization of triple helices in oligodeoxynucleotides,[15,16,20] indicating that the structure of the triple helices formed by DNA–PNA chimeras are different from the triple helices formed by all-DNA oligomers and for this reason, the mechanisms for their stabilization are also different. The results obtained with DNA–PNA chimeras carrying 5-bromouracil and 5-methylcytosine are negative, thus precluding their use for triple helix formation. However, we believe that the results on triple helix formation obtained with DNA–PNA chimeras are encouraging since we have shown that DNA–PNA chimeras bind to duplex DNA better than PNA oligomers. Although PNA oligomers can be designed to form a stable PNA•PNA•DNA triplex via strand-displacement, this process is slow and direct binding to the target duplex DNA may be faster. A more detailed study is needed to determine the structure of the triple helices formed by DNA–PNA chimeras in order to propose new modifications that may enhance the stability of these triple helices.

Table 2. Melting temperatures (Tm) of the triplexes containing DNA–PNA chimeras (100 mM NaCl, 100 mM sodium phosphate/citric acid pH 5.5). PNA part in italics. M = 5-methyl-C; B = 5-bromo-U and $\underline{T}$ = T-hydroxyethylglycine as PNA–DNA linker

Compound	Sequence (5′- > 3′, *N*- > *C*)	Tm (°C)
DNA	CTTCCTCCTCT[a]	38[b]
Chimera I	CTTCC*TCCTCT*[a,c]	24
Chimera II	MTTMM$\underline{T}$*CCTCT*[a,c]	18
Chimera III	CTTCC*T*$\underline{M}$*MTMT*[a,c]	17
Chimera IV	MTTMM$\underline{T}$*MMTMT*[a,c]	21
Chimera V	CTTCC$\underline{T}$*CCBCB*[a,c]	No transition
PNA	Gly-*CTTCCTCCTCT*[a,c]	No transition

[a]Hairpin duplex 5′ GAAGGAGGAGATTTTTCTCCTCCTTC 3′.
[b]Tm = 43 °C, at 1M NaCl, 100 mM sodium phosphate/citric acid pH 5.5.
[c]Terminal 6-(hydroxyhexyl)carboxamide.

Experimental

Materials

MMT-protected PNA monomers of natural bases (N^4-*p-tert*-butylbenzoyl-cytosine; N^6-*p*-methoxybenzoyl-adenine; N^2-acetyl, O^6-diphenylcarbamoyl-guanine and thymine) and *N*-(2-(4-methoxyphenyl)diphenylmethylamino)ethyl-glycine 2-cyanoethyl ester were prepared as previously described.[22] Amino-polyethyleneglycol-polystyrene (PEG-PS) was obtained from PerSeptive Biosystems (USA). Reagents for oligonucleotide synthesis were purchased from Perkin–Elmer-Applied Biosystems (USA) and PerSeptive Biosystems (USA). *N*-Trityl-glycine was from Novabiochem (Switzerland). Solvents were from Merck (Germany) and SDS (France). Chemicals were from Aldrich (USA) and Fluka (Switzerland).

General methods

Flash chromatography was performed using Silica gel 60 (particle size 35–70 μm, SDS, France). During the purification of products having MMT groups the columns were packed with the appropriate mixture containing 1% Et_3N to neutralize the acidity of the silica and the product was eluted with the same mixture without Et_3N. Thin layer chromatography was carried out on aluminum sheets coated with silica gel 60 F_{254} (Merck). Amino compounds were stained with ninhydrin.

MALDI-TOF mass spectrometry measurements of PNA monomers were performed in a Kratos Maldi mass spectrometer using sinapinic acid as the matrix. Electrospray and MALDI-TOF mass spectra of DNA–PNA oligomers were obtained on a Perkin–Elmer AP III SCIEX equipped with a triple-quadrupole detector.

NMR spectra were recorded on a Bruker AM-250 spectrometer. HPLC chomatography was performed on a Waters instrument. For the purification of DNA–PNA oligomers the following conditions were used. Solvent A: 5% ACN in 100 mM triethylammonium acetate (pH 6.5) and solvent B: 70% ACN in 100 mM triethylammonium acetate pH 6.5. For analytical runs: Column, Nucleosil 120C18, 250×4 mm, flow rate: 1 mL/min. Conditions A) a 40 min linear gradient from 0 to 75%B. Conditions (B) a 20 min linear gradient from 0

to 20% B. For preparative runs : Column, PRP-1 (Hamilton), 250×10 mm. Flow rate: 3 mL/min. A 30 min linear gradient from 10 to 80% B (DMT on), or a 30 min linear gradient from 0 to 50% B (DMT off). For the analysis of the PNA monomers the following conditions were used: Column, Nucleosil 120C18, 270×4 mm, flow rate: 1 mL/min. A 30 min linear gradient from 60 to 100% methanol in 0.9% Tris–phosphoric acid pH 2.6.

5-Bromo-1-carboxymethyluracil (1). 5-Bromouracil (2 g, 10 mmol) was dissolved in a KOH solution of 2.23 g (38 mmol) in 8 mL of water. The resulting solution was heated to 40 °C and 2.08 g (15 mmol) of bromoacetic acid dissolved in 4 mL of water was added over a period of 30 min. After the addition, the mixture was stirred at room temperature for 30 min. Conc HCl was then added to the mixture until the pH was 5.5 and the resulting mixture was kept at 0 °C for 2 h. The precipitate was filtered off and the pH of the solution was adjusted to pH 2 with conc HCl. After 2 h at 0 °C, a new precipitate was collected, washed and dried. Yield 1.3 g (50%). TLC (AcOEt: MeOH: AcOH 75:20:5) R_f 0.33. Anal. calcd. for $C_6H_5N_2O_4Br$: C, 28.94; H, 2.02; N, 11.25. Found C, 28.82; H, 2.00; N, 11.09. ^{1}H NMR (d_6-DMSO) δ 4.40 (2H, s), 8.21 (1H, s), 11.89 (1H, s). ^{13}C NMR (d_6-DMSO) δ 48.8,94.6,145.7,150.4,159.7,169.3.

***N*-(2-(4-Methoxyphenyl)diphenylmethylamino)ethyl-*N*-(1-(5-bromouracil) acetyl) glycine 2-cyanoethyl ester (2).** *N*-[2-(4-Methoxyphenyl)diphenylmethylamino]ethyl-glycine 2-cyanoethyl ester (3.3 g, 7.4 mmol) was dissolved in DMF (15 mL) and 1.87 mL (14.7 mmol) of *N*-ethylmorpholine and 1.19 g (7.4 mmol) of HOOBt were added to the solution, followed by a solution of 1.83 g (7.4 mmol) of 5-bromo-1-carboxymethyluracil in 10 mL of DMF and 1.11 mL (8.8 mmol) of DIP. The resulting mixture was stirred at room temperature for 20 h and then concentrated to dryness. The residue was dissolved in DCM and the solution was washed with water and saturated NaCl aqueous solution. The organic phase was dried and concentrated to dryness. The resulting product was purified on silica gel. The column was packed with DCM containing 1% Et_3N and eluted with 0–2% MeOH gradient in DCM. The desired product was obtained as a yellowish oil (2.9 g, 68%) partially contaminated with diisopropylurea (HPLC 85% pure, 14.3 min). TLC (3% MeOH in DCM) R_f 0.35 . ^{1}H NMR ($CDCl_3$) δ 2.37 (2H, m), 2.61 (2H, t, J = 6.2 Hz), 3.54 (2H, m), 3.81 (3H, s), 4.02 (2H, s), 4.23 (2H, t, J = 6.2 Hz), 6.80–7.51 (14H, m), 7.87 (1H, s). ^{13}C NMR ($CDCl_3$) δ 17.7, 41.9, 48.3, 48.7, 50.1, 55.2, 59.4, 70.6, 96.5, 113.2, 116.7, 126.3, 127.5, 128.3, 129.6, 137.5, 144.5, 145.7, 150.3, 158.0, 160.0, 167.2, 168.4. MS (MALDI-TOF): Found 675 (M + 1); 273 (MMT+); (molecular mass expected for $C_{33}H_{32}N_5O_6Br$ 674.5).

***N*-(2-(4-Methoxyphenyl)diphenylmethylamino)ethyl-*N*-((5-bromouracil) acetyl) glycine (3).** Compound **2** (2.9 g, 4.3 mmol) was treated with 52 mL of a 0.5 M DBU solution (26 mmol) in acetonitrile at room temperature. After 5 min, TLC (5% MeOH/DCM) showed complete hydrolysis of the ester. The reaction mixture was concentrated to dryness. The product was further purified on silica gel. The column was packed with a 3% MeOH/DCM solution containing 1% of triethylamine and the product was eluted with a 3–8% MeOH gradient in DCM. The desired product was obtained as triethylammonium salt. Yield 1.79 g (58%). (HPLC > 90%, 11.5 min). TLC (5% MeOH in DCM) R_f 0.13. ^{1}H NMR ($CDCl_3$) δ 1.24 (t, Et_3N), 2.35 (2H, m), 2.97 (q, Et_3N), 3.54 (2H, m), 3.95 (2H, s), 4.60 (2H, s), 3.78 (3H, s), 7.10–7.58 (14H, m), 7.70 (1H, s). ^{13}C NMR ($CDCl_3$): δ (major rotamer) 8.5 (Et_3N), 41.8, 45.2 (Et_3N), 47.8, 48.7, 52.2, 55.1, 70.3, 95.8, 113.0, 126.2, 127.7, 128.5, 129.7, 138.0, 144.8, 145.2, 150.4, 157.7, 159.8, 167.2, 173.7. MS (MALDI-TOF): Found 620 (M); 273 (MMT+); (molecular mass expected for $C_{33}H_{32}N_5O_6Br$ 620.4)

1-*tert*-Butoxycarbonylmethylthymine (4). Thymine (3 g, 23.8 mmol) was dissolved in 70 mL of DMF and 3.9 mL of DBU was added. 3.5 mL (35.7 mmol) of ethyl bromoacetate dissolved in 3 mL of DMF was added dropwise to the solution. After stirring for 1 h at room temperature, the solvents were evaporated and the residue was dissolved in AcOEt. The organic phase was washed twice with a phosphate solution pH 7 followed by a saturated NaCl aqueous solution. The organic phase was dried and concentrated to dryness. The resulting product was purified on silica gel using a 1–2% MeOH gradient in DCM. Yield 4.64 (81%). TLC (4% MeOH in DCM) R_f 0.48. ^{1}H NMR ($CDCl_3$) δ 1.1 (9H, s), 1.8 (3H, s), 4.2 (2H, s), 7.4 (1H, s). ^{13}C NMR ($CDCl_3$): δ 12.3, 27.9, 49.1, 83.4, 110.9, 140.3, 150.9, 164.2, 166.6.

1-*tert*-Butoxycarbonylmethyl-O^4-(*o*-nitrophenyl)thymine (5). 1-*tert*-Butoxycarbonylmethylthymine (5.1 g, 21 mmol) was dissolved in 100 mL of DMF. To the solution 15 mL (100 mmol) of Et_3N, 6.9 g (31.5 mmol) of 2-mesitylenesulfonyl chloride and 0.64 g (8.4 mmol) of DMAP were added. After stirring for 3 h at room temperature 0.47 g (10.5 mmol) of 1,4-diazabicyclo[2.2.2]octane and 3.5 g (25.2 mmol) of *o*-nitrophenol were added and the solution was stirred for 20 h. The reaction mixture was then added to a 1M $NaHCO_3$ aqueous solution (300 mL) with stirring. The organic phase was collected, dried and concentrated to dryness. The residue was dissolved in hot isopropanol and cooled. The white precipitate thus obtained was further purified by silica gel column chromatography using a 0–4% MeOH gradient in DCM. Yield 4.6 g (64%). TLC (2% MeOH in DCM) R_f 0.22. ^{1}H NMR ($CDCl_3$) δ 1.12 (9H, s), 1.8 (3H, s), 4.11 (2H, s), 6.9–7.3 (4H, m), 7.8 (1H, d). ^{13}C NMR ($CDCl_3$): δ 12.1, 27.9, 50.9, 83.2, 104.3, 125.8, 126.6, 134.9, 141.5, 145.3, 146.5, 155.5, 166.6, 170.1.

1-*tert*-Butoxycarbonylmethyl-5-methylcytosine (6). Compound **5** (8 g, 23.2 mmol) was dissolved in 300 mL of THF. The solution was treated with an excess of liquid ammonia (22 g, 1.29 mol) in a steel bomb. The stirred (500 rpm) mixture was heated to 75 °C for 4 days. The mixture was concentrated to dryness. The residue was purified by silica gel chromatography. The column was packed with 1% Et_3N in a MeOH-DCM (3:97) mixture and eluted with 3% MeOH in DCM. Yield 3.5 g (68%). TLC (5% MeOH in DCM + 1% Et_3N) R_f 0.19. ^{1}H NMR (d_6-DMSO) δ 0.98 (9H, s), 1.40 (3H, s), 3.87 (2H,

s), 6.95 (1H, s). ^{13}C NMR (d_6-DMSO): δ 12.8, 27.7, 50.2, 81.1, 100.6, 143.8, 155.7, 165.8, 167.8.

N^4-Benzoyl-1-*tert*-butoxycarbonylmethyl-5-methylcytosine (7). Compound **6** (0.34 g, 1.5 mmol) was dissolved in 20 mL of pyridine and 0.41 g (1.82 mmol) of benzoic anhydride were added. After magnetic stirring for 16 h at room temperature 2 mL of water was added and the mixture was concentrated to dryness. The residue was dissolved in DCM and washed with 1 M $NaHCO_3$ aqueous solution. The organic phase was dried and concentrated to dryness. The residue was purified by silica gel chromatography and the product was eluted with a 0–5% MeOH gradient in DCM. Yield 0.32 g (65%). TLC (2% MeOH in DCM) R_f 0.37. Anal. calcd for $C_{18}H_{21}N_3O_4$: C, 62.96; H, 6.16; N, 12.24. Found C, 62.64; H, 6.17; N, 11.86. ^{1}H NMR ($CDCl_3$) δ 1.48 (9H, s), 2.11 (3H, s), 4.21 (2H, s), 7.09 (1H, s), 7.41–8.33 (5H, m). ^{13}C NMR ($CDCl_3$): δ 13.2, 27.9, 49.8, 83.4, 111.7, 128.0, 129.8, 132.4, 137.1, 141.6, 148.4, 166.3, 179.5, 160.2.

N^4-Benzoyl-1-carboxymethyl-5-methylcytosine (8). Compound **7** (0.32 g, 1.0 mmol) was treated with 5 mL of TFA. After 40 min of magnetic stirring, the reaction was completed by TLC. Ethyl ether (20 mL) was added and the white precipitate was filtered and dried. Yield : 0.3 g (72%). TLC (5% MeOH in DCM) R_f 0.28. ^{1}H NMR (d_6-DMSO) δ 2.08 (3H, s), 4.60 (2H, s), 7.4–8.4 (6H, m). ^{13}C NMR ($CDCl_3$): δ 12.8, 49.5, 108.9, 128.3, 129.2, 132.5, 136.1, 145.3, 149.0, 160.5, 169.1.

***N*-(2-(4-Methoxyphenyl)diphenylmethylamino)ethyl-*N*-((*N*^{4}-benzoyl-5-methylcytosin-1-yl)acetyl) glycine 2-cyanoethyl ester (9).** *N*-[2-(4-Methoxyphenyl)diphenylmethylamino]ethyl-glycine 2-cyanoethyl ester (1.1 g, 2.5 mmol) was reacted with N^4-benzoyl-1-carboxymethyl-5-methylcytosine (0.7 g, 2.5 mmol) in the presence of *N*-ethylmorpholine (0.64 mL, 5.1 mmol), HOOBt (0.4 g, 2.5 mmol) and DIP (0.38 mL, 3.1 mmol) as described for compound **2**. The resulting product was purified on silica gel. The column was packed with 1% Et_3N in DCM and the product was eluted with DCM and then with DCM: AcOEt (4:1). The desired product was obtained as a yellowish oil (1.3 g, 74%) partially contaminated with diisopropylurea (HPLC 85% pure, 23.3 min). R_f = 0.41 (DCM:AcOEt 3:1). ^{1}H NMR ($CDCl_3$): δ 1.17 (3H, s), 2.38 (2H, m), 2.62 (2H, t, J = 6.2 Hz), 3.60 (2H, m), 3.80 (3H, s), 4.10 (2H, s), 4.21 (2H, t, J = 6.2 Hz), 5.20 (2H, s), 6.83–7.53 (15H, m), 8.31 (1H, s). ^{13}C NMR ($CDCl_3$): δ 13.4, 17.8, 42.1, 48.7, 48.8, 50.3, 55.2, 59.4, 70.6, 111.7, 113.2, 126.4, 127.9, 128.0, 128.4, 128.6 129.7, 132.2, 137.5, 142.2, 145.7, 148.7, 158.1, 160.3, 166.4, 168.4. MS (MALDI-TOF): Found 711 (M); 273 (MMT+); (molecular mass expected for $C_{41}H_{40}N_6O_6$ 712.8).

***N*-(2-(4-Methoxyphenyl)diphenylmethylamino)ethyl-*N*-((*N*^{4}-benzoyl-5-methylcytosin-1-yl)acetyl) glycine (10).** Compound **9** (1.3 g, 1.9 mmol) was treated with 22 mL of a 0.5 M DBU solution in acetonitrile (11.2 mmol) at room temperature for 5 min as described for compound **3**. During the purification of this compound the product was eluted with 5–8% MeOH gradient in DCM. Yield 0.85 g (72%) (HPLC > 98%, 21.4 min). R_f = 0.25 (10% MeOH in DCM). ^{1}H NMR ($CDCl_3$): δ 1.21 (Et_3N), 2.10 (3H, s), 2.37 (2H, t, J = 5.5 Hz), 2.85 (Et_3N), 3.56 (2H, t, J = 5.5 Hz), 3.79 (3H, s), 3.93 (2H, s), 4.64 (2H, s), 6.81–7.56 (14H, m), 8.34 (1H, s).^{13}C NMR ($CDCl_3$): δ (major rotamer) 8.9 (Et_3N), 13.3, 41.9 , 45.2 (Et_3N), 48.4, 49.3, 50.6, 55.1, 70.3, 110.8, 113.1, 126.4, 127.8, 120.0, 128.4, 128.5, 129.8, 132.2, 137.5, 142.9, 145.7, 157.9, 166.5, 173.5. MS (MALDI-TOF): found 659 (M), 388 (M-MMT + 2H) (M), 273 (MMT+); (molecular mass expected for $C_{41}H_{43}N_5O_6$ 658.7).

Syntheses of DNA–PNA chimeras and PNA oligomer

DNA–PNA hybrids were synthesized on an MMT-NH-hexyl-succinate-NH-PEG-PS-support as described in ref 5. Sequences were I: 5′ CTTCC*T̲CCTCT*-CONH-hexyl-OH, II: 5′ MTTCCMM*T̲CCT̲CT*-CONH-hexyl-OH, III: 5′ CTTCC*T̲MMTMT̲*-CONH-hexyl-OH, IV: 5′ MTTMM*T̲MMT̲MT*-CONH-hexyl-OH and V: 5′ CTTCC*T̲CCB̲CB*-CONH-hexyl-OH where M represents 5-methylcytosine, B is 5-bromouracil and *T̲* is a PNA monomer carrying a hydroxyl group instead of an amino group.[6,10] The PNA part was synthesized at 10 μmol scale in a syringe equipped with a filter. The PNA monomers were added manually in a 5-fold molar excess. PNA monomer coupling was carried out by adding equal amounts of 3 solutions : (a) 0.2 M PNA monomer in ACN (T, G, A) or 0.1 M of the C monomer in DCM (C), (b) 0.2 M HATU in ACN and (c) 0.2 M DIPEA in ACN and a coupling time of 15 min. Capping was performed by a 1 min-treatment of the supports with the acetic anhydride and *N*-methylimidazole solutions used on oligonucleotide synthesis. The monomethoxytrityl groups were deprotected using 3% TCA in DCM (10×1 mL, 5 min) followed by thorough washing with ACN, neutralization with 0.3 M DIPEA in ACN and washing with ACN. The last PNA monomer is a T-linker molecule carrying an OH protected with the MMT group.[6,10] The oligonucleotide part was assembled on an automatic DNA synthesizer using standard 2-cyanoethyl phosphoramidites and following standard protocols (1 μmol scale, DMT on). After the assembly of the sequences, oligonucleotide-supports were treated with 32% aqueous ammonia at 55 °C for 16 h except for sequence V, which was treated with concentrated ammonia at room temperature for 24 h. Ammonia solutions were concentrated to dryness and the products were purified by reversed phase HPLC using DMT on and DMT off protocols. All purified products presented a major peak which was collected and analyzed by mass spectrometry. Mass spectra (electrospray) : sequence I: found 3146.3 (expected for $C_{116}H_{157}N_{40}O_{55}P_5$: 3146.2); sequence II: found 3188.5 (expected for $C_{119}H_{163}N_{40}O_{55}P_5$: 3188.3); sequence III: found 3188.0 (expected for $C_{119}H_{163}N_{40}O_{55}P_5$: 3188.3); sequence IV: found 3229.7 (expected for $C_{122}H_{169}N_{40}O_{55}P_5$: 3230.4); sequence V: found 3276.5 (expected for $C_{114}H_{151}N_{40}O_{55}P_5Br_2$: 3276.2). Yields (optical density units at 260 nm after HPLC purification, 1 μmol) were between 20 and 30.

The PNA oligomer Gly-*CTTCCTCCTCT*-CONH-hexyl-OH was prepared as described above for the PNA

part of the DNA–PNA chimeras. After the assembly of the PNA sequence *N*-trityl-glycine was coupled following the protocol described for the PNA monomers leaving the last trityl group on the sequence. Deprotection and purification were carried out as described for DNA–PNA chimeras. Mass spectra (electrospray): found 3012.0 (expected for $C_{123}H_{166}N_{52}O_{40}$: 3012.9).

Melting experiments

Melting experiments of duplexes were carried out by mixing equimolar amounts of two complementary strands dissolved in a solution containing 0.15 M NaCl, 0.05 N Tris–HCl buffer pH 7.5. Duplexes were annealed by slow cooling from 80 to 4 °C. UV absorption spectra and melting curves (absorbance versus temperature) were recorded in 1-cm path-length cells using a Varian Cary 13 spectophotometer having a temperature controller with a programmed temperature increase of 0.5 °C/min. Melts were run on duplex concentrations of 4 μM at 260 nm.

Melting experiments with triple helix were performed as follows: solutions of equimolar amounts of the hairpin oligonucleotide (h_{26}) and the appropriate 11-mer were mixed in the appropriate buffer. The solutions were heated to 80 °C, allowed to cool slowly to room temperature and then kept in the refrigerator overnight. UV absorption spectra and melting experiments (absorbance versus temperature) were recorded in 1-cm path-length cells using a spectrophotometer, which has a temperature controller with a programmed temperature increase of 0.5 °C/min. Melts were run on triplex concentration of 4 μM at 270 nm.

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References and Notes

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