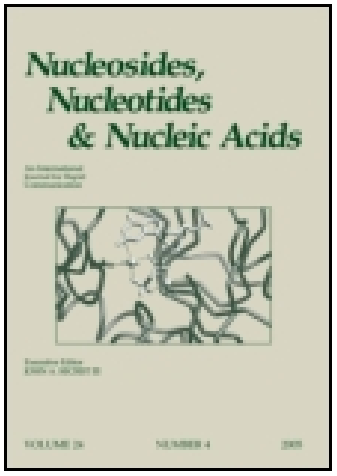

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Synthesis and Nucleic Acids Binding Properties of Diastereomeric Aminoethylprolyl Peptide Nucleic Acids (aepPNA)

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SYNTHESIS AND NUCLEIC ACIDS BINDING PROPERTIES OF DIASTEREOMERIC AMINOETHYLPROLYL PEPTIDE NUCLEIC ACIDS (*aep*PNA)

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□ *A general synthetic method for Fmoc-protected monomers of all four diastereomeric aminoethyl peptide nucleic acid (*aep*PNA) has been developed. The key reaction is the coupling of nucleobase-modified proline derivatives and Fmoc-protected aminoacetaldehyde by reductive alkylation. Oligomerization of the* aep*PNAs up to 10mer was achieved by Fmoc-solid phase peptide synthesis methodology. Preliminary binding studies of these* aep*PNA oligomers with nucleic acids suggested that the "*cis*-" homothymine* aep*PNA decamers with (2′*R*,4′*R*) and (2′*S*,4′*S*) configurations can bind, albeit with slow kinetics, to their complementary RNA [poly(adenylic acid)] but not to the complementary DNA [poly(deoxyadenylic acid)]. On the other hand, the* trans *homothymine* aep*PNA decamers with (2′*R*,4′*S*) and (2′*S*,4′*R*) configurations failed to form stable hybrid with poly(adenylic acid) and poly(deoxyadenylic acid). No hybrid formation could be observed between a mixed-base (2′*R*,4′*R*)-*aep*PNA decamer with DNA and RNA in both antiparallel and parallel orientations.*

Keywords PNA; RNA; hybridization; triplex forming oligonucleotides

INTRODUCTION

Peptide nucleic acid is a structural mimic of DNA that exhibits interesting base-pairing properties.[1] Since the first introduction of aminoethylglycyl (*aeg*) PNA in 1991,[2] a number of modifications of the original *aeg*PNA backbone have been proposed with the aim to improve certain properties such as binding affinity, solubility and cell penetration. A considerable success has been made by restriction of the original *aeg*PNA conformations by incorporating one or more ring structures in the backbone.[3,4] Some of these conformationally restricted PNA exhibited comparable or even

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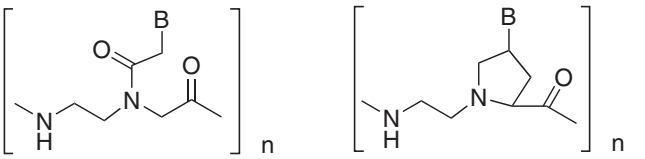


*aeg*PNA *aep*PNA

FIGURE 1 Structures of *aeg*PNA and *aep*PNA (stereochemistry not shown).

stronger affinity to DNA and/or RNA, while the high specificity of the base-pairing was still retained. Furthermore, few modified PNA even possess extra features not present in the original Nielsen's *aeg*PNA such as preference for binding to DNA in one specific direction and preferential binding to specific types of nucleic acids (DNA or RNA)[5–7].

Since 1999, at least three research groups have independently reported syntheses and hybridization studies of a new conformationally constrained and positively charged PNA known as aminoethylprolyl PNA (*aep*PNA, Figure 1). The presence of a positive charge on the non-acylated pyrrolidine nitrogen atom ($pK_a \sim 6.5$)[8] in the PNA backbone could potentially improve the solubility and binding affinity to DNA, which is negatively charged. D'Costa and coworkers have studied two of the four possible stereoisomers of *aep*PNA. They reported that the homothymine *aep*PNA octamer with (2′*S*,4′*S*) and (2′*R*,4′*S*) stereochemistry formed very stable $(PNA)_2$·DNA triplexes with their complementary DNA ($T_m > 80°C$).[8] They also reported a site-specific incorporation of *aep*PNA monomers into Nielsen's *aeg*PNA oligomers by solid phase synthesis. These chimeric *aeg*-*aep*PNAs were shown to have improved antiparallel *vs* parallel directional selectivity toward DNA compared to the unmodified *aeg*PNA, while the binding affinity and specificity was retained.[9,10] Liu and coworkers reported the synthesis of nonchimeric *aep*PNA oligomers with (2′*S*,4′*S*) stereochemistry.[11] In contrast to earlier findings,[8] The non-chimeric homothymine and mixed-sequence *aep*PNA showed no detectable binding with complementary DNA according to UV melting and titration.[11] Our study on *aep*PNA oligomer with a different stereochemistry, namely (2′*R*,4′*R*), revealed that the homothymine *aep*PNA decamer forms a fairly stable hybrid with complementary RNA, whereas no binding to the complementary DNA could be observed.[12]

The hybridization properties of *aep*PNA oligomers reported so far showed some discrepancies, which may be sequence and stereochemistry dependent. None of these research groups has yet synthesized all four possible stereoisomers and perform a systematic comparison under identical conditions. In addition, work from other groups involving *aep*PNA mostly emphasized on its DNA binding properties. In view of our previous success in developing a PNA that binds preferentially to DNA,[5,6] it would be desirable to have an alternative PNA system that binds favorably, or at least

equally well, to RNA. These RNA binding PNAs would have a great potential in antisense applications.[1] Toward this goal, a general synthetic route toward the PNA momomers and oligomers first needs to be developed. In this paper, we report a successful synthesis of all four stereoiosomers of Fmoc-protected *aep*PNA monomers employing a new reductive alkylation strategy. We also report a synthesis of the *aep*PNA up to 10mer from these monomers as well as a preliminary binding study between these diastereomeric *aep*PNA oligomers and DNA/RNA.

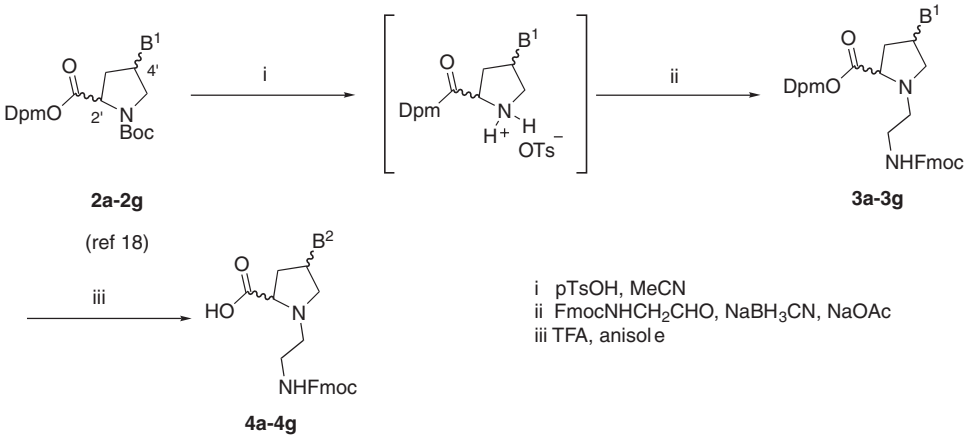
RESULTS AND DISCUSSION

Synthesis of Monomers

Ganesh[8,9] and Lui[11] had previously developed a method for the synthesis of Boc-protected *aep*PNA monomers. *N*-alkylation of 4-hydroxyproline esters with *N*-Boc-bromoethylamine afforded *N*-(*N*-Boc-aminoethyl)hydroxyproline intermediates in 60–68% yield. These hydroxyl compounds were activated by mesylation and then displaced with an appropriately protected nucleobase to give the *N*-Boc protected *aep*PNA monomers in moderate yields. In our previous report, a different approach had been taken whereby a nucleobase-modified proline was *N*-alkylated with *N*-nosylaziridine.[12] Exchanging the *N*-protecting groups from nosyl to Fmoc via the Boc-protected intermediate provided the expected monomers. These methods, however, suffer from providing low yield and require many steps.

Reductive alkylation of amino acids by *N*-protected aminoaldehydes in the presence of $NaBH_3CN$ had been widely used in the synthesis of *N*-aminoethylated amino acids, including *aeg*PNA monomers.[13–16] We proposed that the reductive alkylation of nucleobase-substituted proline with *N*-Fmoc-aminoacetaldehyde should directly provide the Fmoc-protected *aep*PNA monomers without the need for protecting group conversion (Scheme 1).

N-Fmoc-aminoacetaldehyde (**1**) was easily prepared in two steps with 90% overall yield from $NaIO_4$ oxidation of 3-amino-1,2-propanediol.[16,17] The Boc group in the *N*-Boc thymine-modified proline derivative (**2a**) was selectively removed in the presence of the diphenylmethyl ester by treatment with 3 equivalents of *p*-toluenesulfonic acid (*p*-TsOH) in acetonitrile at ambient temperature.[18] The reductive alkylation of the free amine with **1** took place in the presence of $NaBH_3CN$ as a reductant and NaOAc as a buffering agent. The desired product (**3a**) was obtained as a white foam in 76% overall yield (two steps from **2a** and **1**). Spectroscopic data of the product were identical to those of the same compound obtained *via* alkylation with aziridine followed by protecting group conversion.[12] Other Boc-protected



SCHEME 1 Synthesis of *aep*PNA monomers via reductive alkylation.

nucleobase-modified proline derivatives (**2b–2g**)[18] were then subjected to the same reductive alkylation with **1** under similar conditions. In all cases the expected products **3b–3g** were obtained in moderate to good yields (Table 1). Although no satisfactory explanation could be offered, it is interesting to note that the reductive alkylation of the "*trans*" (2′*R*,4′*S*) and (2′*S*,4′*R*) isomers consistently gave poorer yield compared to the "*cis*" (2′*R*,4′*R*) and (2′*S*,4′*S*) isomers (Table 1, compare entries 3, 4, and 1, 2).

To obtain the Fmoc-protected free acids **4**, the diphenylmethyl ester group in **3a–3g** was next removed by treatment with trifluoroacetic acid in the presence of anisole at ambient temperature. The free acids **4a–4g** were obtained in 72–85% yield (Table 1). In case of the thymine monomers **4a–4d**, the N^3-benzoyl group on the thymine was simultaneously cleaved under these conditions. As protection of the thymine-N^3 was not generally required during the PNA synthesis, this premature cleavage would not be considered as a serious problem.

TABLE 1 Synthesis of protected PNA monomers by reductive alkylation with *N*-Fmoc-aminoacetaldehyde

Entry	Starting material	Config.	Product	B^1	Yield (%)	Product	B^2	Yield (%)
1	**2a**	(2′*R*,4′*R*)	**3a**	T^{Bz}	76	**4a**	T	85
2	**2b**	(2′*S*,4′*S*)	**3b**	T^{Bz}	87	**4b**	T	72
3	**2c**	(2′*R*,4′*S*)	**3c**	T^{Bz}	40	**4c**	T	72
4	**2d**	(2′*S*,4′*R*)	**3d**	T^{Bz}	43	**4d**	T	75
5	**2e**	(2′*R*,4′*R*)	**3e**	G^{Ibu}	69	**4e**	G^{Ibu}	77
6	**2f**	(2′*R*,4′*R*)	**3f**	A^{Bz}	60	**4f**	A^{Bz}	75
7	**2g**	(2′*R*,4′*R*)	**3g**	C^{Bz}	89	**4g**	C^{Bz}	74

Epimerization at the position 2′ in the proline ring *via* the enamine intermediate resulting from iminium-enamine tautomerization would have been considered as a plausible side reaction of the present reductive alkylation strategy. If this had taken place, a diastereomeric mixture consisting of the (2′*R*,4′*R*) and (2′*S*,4′*R*) products would have formed. ^{1}H and ^{13}C NMR spectra of the compounds **3** and **4** suggested the presence of only one diastereomer in all cases. Furthermore, HPLC analysis of the compound **4a**, which possesses (2′*R*,4′*R*) stereochemistry, revealed no detectable contamination by the (2′*S*,4′*R*) diastereomer **4d** (<0.5%, verified by co-injection t_R *RR* = 46.4 minutes, t_R *SR* = 47.2 minutes). This confirmed that no significant epimerization had occurred during the reductive alkylation and deprotection steps.

The present reductive alkylation of nucleobase-derivatized proline proved to be a more efficient route for the synthesis of *aep*PNA monomers than the previously reported methods. It provided the Fmoc-protected intermediates **4a–4g** directly without the need for protecting group conversion. The Fmoc-protected intermediate **3a** ($B^1 = T^{Bz}$) has previously been obtained in 22% overall yield (6 steps) starting from **2a**,[12] while the present method gave the desired products in 40–89% overall yield (two steps) depending on the nucleobases.

Synthesis of *aep*PNA Oligomers

Oligomerization of the activated *aep*PNA monomers was performed by manual Fmoc-solid phase peptide synthesis methodology.[19] The sequences synthesized included four homothymine *aep*PNA decamers with different configurations at the proline moiety (denoted ***RR*-T$_{10}$**, ***SS*-T$_{10}$**, ***RS*-T$_{10}$** and ***SR*-T$_{10}$** in Table 2), and a mixed base decamer with (2′*R*,4′*R*) configurations (denoted ***RR*-mix** in Table 2). All sequences were capped with acetyl and lysine amide at the N- and C-termini respectively. Generally, the average coupling yields of 95–99% for each step were achieved according to UV absorbance measurement of the dibenzofulvene-piperidine adduct released during deprotection step. In cases of sequences containing nucleobases

TABLE 2 Sequences and characterization details of the *aep*PNA synthesized

Entry	Code	Config.	Sequence[a]	M·H$^+$ (calcd.)	M·H$^+$ (found)[b]
1	***RR*-T$_{10}$**	(2′*R*,4′*R*)	TTTTTTTTTT	2830.05	2830.04
2	***SS*-T$_{10}$**	(2′*S*,4′*S*)	TTTTTTTTTT	2830.05	2830.27
3	***SR*-T$_{10}$**	(2′*S*,4′*R*)	TTTTTTTTTT	2830.05	2830.31
4	***RS*-T$_{10}$**	(2′*R*,4′*S*)	TTTTTTTTTT	2830.05	2830.24
5	***RR*-mix**	(2′*R*,4′*R*)	GTAGATCACT	2877.09	2877.57

[a]*N*-Ac, *C*-LysNH_2 capped.
[b]MALDI-TOF, α-cyano-4-hydroxy cinnamic acid (CCA) matrix.

other than thymine, the nucleobase protecting groups (Bz, Ibu) were removed after completion of the synthesis by treatment of the support-bound PNA with aqueous ammonia/dioxane 1:1 at 60°C for 6 hours prior to the usual TFA cleavage. The decamer *aep*PNAs were obtained after reverse phase HPLC purification and were characterized by MALDI-TOF mass spectrometry. In all cases quasi-molecular ions were observed with m/z within ±0.5 Da to the calculated values, thus confirming the identities of all PNA synthesized (Table 2).

DNA and RNA Binding Properties of *aep*PNA

Interactions between the decamer *aep*PNA with complementary DNA and RNA were investigated by T_m measurement. The thermal transition curves were recorded at 260 nm over 20–90°C range and the T_m values are shown in Table 3.

The binding experiments clearly indicate that the homothymine *aep*PNA decamers with *cis*-configurations (***RR*-T$_{10}$** and ***SS*-T$_{10}$**) could form reasonably stable hybrids with poly(rA) (T_m = 43 and 42°C) but not poly(dA). On the other hand, the homothymine *aep*PNA decamers with *trans*-configurations (***RS*-T$_{10}$** and ***SR*-T$_{10}$**) failed to form stable hybrids with both poly(rA) and poly(dA), although an apparent melting was observed between ***RS*-T$_{10}$** and poly(dA) at a rather low temperature (24°C). CD spectra of the *aep*PNA and their mixture with their complementary DNA/RNA were in good agreement with T_m data. Significant differences between the observed and sum of the individual component CD spectra, which indicate hybrid formation, were observed only for the pairs ***RR*-T$_{10}$**/poly(rA) and ***SS*-T$_{10}$**/poly(rA) (Figure 2). Disappointingly, no hybridization of the mixed base *aep*PNA decamer (***RR*-mix**) to complementary DNA and RNA could be observed in both parallel

TABLE 3 T_m values of hybrids between decamer *aep*PNA and polynucleotides

Entry	*aep*PNA	polynucleotide	T_m (°C)[a]
1	***RR*-T$_{10}$**	poly(rA)	43
2	***SS*-T$_{10}$**	poly(rA)	42
3	***SR*-T$_{10}$**	poly(rA)	<20
4	***RS*-T$_{10}$**	poly(rA)	<20
5	***RR*-T$_{10}$**	poly(dA)	<20
6	***SS*-T$_{10}$**	poly(dA)	<20
7	***SR*-T$_{10}$**	poly(dA)	<20
8	***RS*-T$_{10}$**	poly(dA)	24
9	***RR*-mix**	d(CATCTAGTGA)	<20
10	***RR*-mix**	d(AGTGATCTAC)	<20
11	***RR*-mix**	r(CAUCUAGUGA)	<20
12	***RR*-mix**	r(AGUGAUCUAC)	<20

[a] T_m were measured at [PNA] = [DNA] = 1 μM, 10 mM sodium phosphate pH 7.0, heating rate 1°C/min.

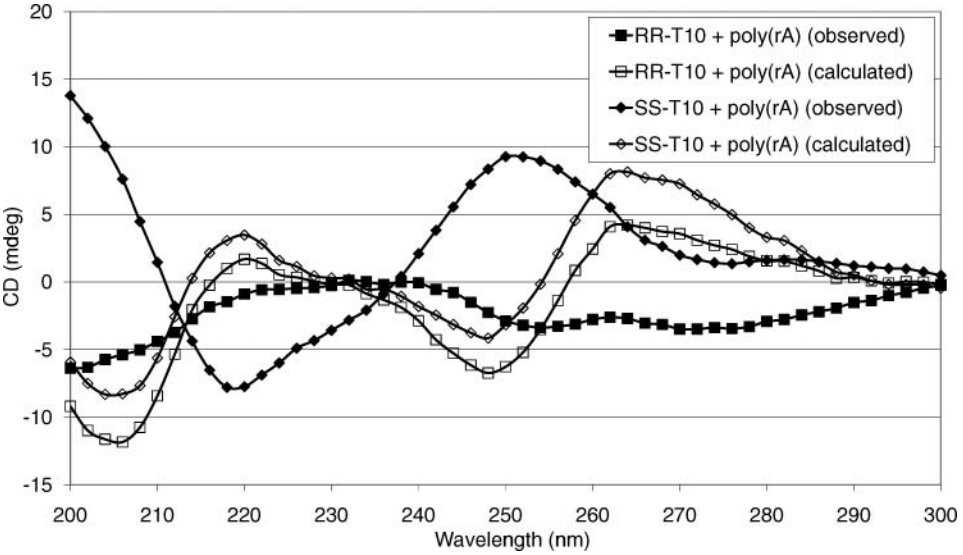


FIGURE 2 CD spectra of PNA-DNA or RNA hybrids. Conditions: [PNA] = [DNA or RNA] = 1 μM, 10 mM sodium phosphate pH 7.0, 25°C.

(Table 3, entries 9 and 11) and antiparallel orientations (Table 3, entries 10 and 12). The duplex structures formed between the mixed base *aep*PNA with (2′*R*,4′*R*) configuration is therefore likely to be less stable than the corresponding DNA·DNA or DNA·RNA hybrids.[20] The unusually high stability of the homothymine *aep*PNA hybrids compared to the mixed sequence in this case could be explained by the formation of triplex structures, which was confirmed by ultraviolet (UV) titration experiments. It had been previously observed in other PNA systems with homopyrimidine sequences that these triplexes could form exclusively even when the stoichiometry was not correct because the triplex was more stable than the intermediate duplex.[1] Our results with the (2′*S*,4′*S*) isomer is in accordance with the observation by Liu that no stable hybrid was formed between homothymine *aep*PNA and its complementary DNA.[11] Preliminary time-dependent UV experiments indicated that the binding between the *aep*PNA (***RR*-$\mathbf{T_{10}}$** and ***SS*-$\mathbf{T_{10}}$**) and RNA are kinetically slow. More than 30 minutes are required for the maximum hypochromism to be established (Figure 3). The significant hysteresis observed in the cooling curves of both *aep*PNA-RNA hybrids also confirmed the slow kinetics of the hybridization, which may suggest that there was substantial reorganization of the single stranded counterparts upon hybridization. No hypochromism was observed in the corresponding DNA experiments, confirming that both *aep*PNAs could not bind to their complementary DNA under these conditions. Whether the preferential binding of the *aep*PNA to RNA, and the failure of the mixed base *aep*PNA to bind to both DNA and

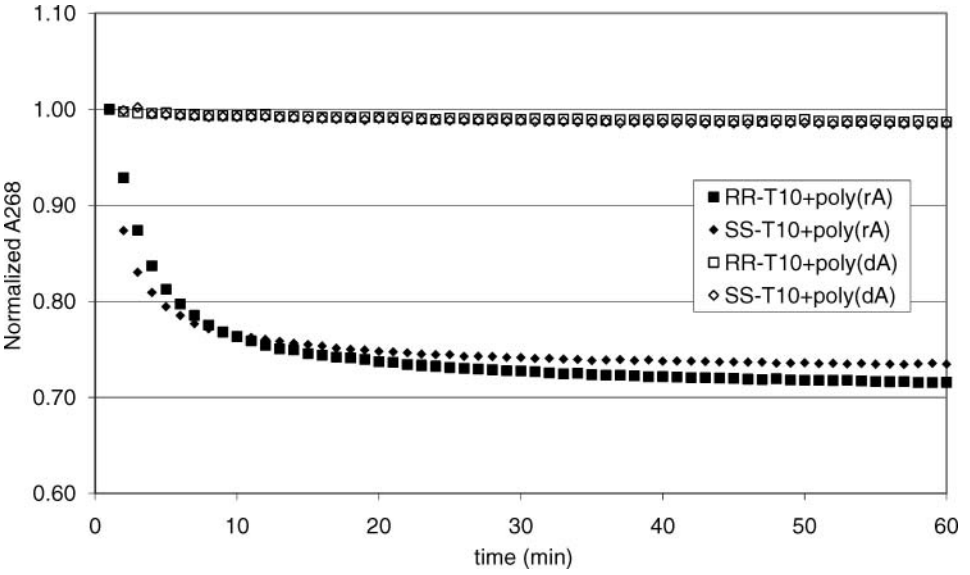


FIGURE 3 Time-dependent normalized UV absorbance at 268 nm of *aep*PNA-DNA/RNA hybridization reactions. Conditions: [PNA] = [DNA or RNA] = 1 μM, 10 mM sodium phosphate pH 7.0, 25°C.

RNA are due to thermodynamic or kinetic factors, and the structural basis of this selectivity, remains to be further investigated.

CONCLUSION

The reductive alkylation of nucleobase-modified proline derivative with Fmoc-aminoacetaldehyde (**1**) was found to be an efficient method for the synthesis of *aep*PNA monomers. Deprotection of the C-terminal protecting group gave the (2′*R*,4′*R*)-*aep*PNA monomers containing all four nucleobase (A^{Bz}, T, C^{Bz}, and G^{Ibu}) and the thymine monomers with different configuration (2′*R*,4′*R*, 2′*S*,4′*S*, 2′*R*,4′*S*, 2′*S*,4′*R*). Oligomerization of the PNA monomers was carried out employing Fmoc SPPS. Five *aep*PNA decamers were successfully synthesized. The hybridization properties of the *aep*PNA were investigated by UV and circular dichroism (CD) spectroscopy. The results suggested that the stereochemistry on the pyrrolidine ring and the nucleobase sequence can have a dramatic effect on the binding characteristics of the *aep*PNA. Only the *cis*-stereoisomers with a homothymine sequence can form stable triplexes with RNA, but not DNA. The hybrid formation in these cases is kinetically slow. No hybrid formation could be observed between a mixed-base sequence (2′*R*,4′*R*)-*aep*PNA with DNA and RNA in both antiparallel and parallel orientations.

EXPERIMENTAL

Materials and Methods

^{1}H and ^{13}C spectra were recorded on a Varian Mercury-400 plus NMR spectrometer operating at 400 MHz. MALDI-TOF mass spectra were obtained in reflectron mode on an Omniflex MALDI-TOF mass spectrometer (Bruker Daltonics, Germany) using α-cyano-4-hydroxy cinnamic acid (CCA) as matrix employing 0.1% trifluoroacetic acid in acetonitrile:water (1:2) as diluents for samples preparation. CD experiments were performed on a JASCO Model J-810 Spectropolarimeter, Japan. The CD spectra were recorded at 25°C from 300 to 200 nm and averaged four times then subtracted from a spectrum of the buffer under the same conditions. All chemicals were purchased from Fluka (Switzerland), Merck (Germany), or Aldrich (USA), and were purified as appropriate. The *N-tert*-butoxycarbonyl (Boc)/diphenylmethyl (Dpm) ester protected nucleobase-modified proline derivatives **2a–2d** were synthesized from *trans*-4-hydroxy-L-proline according to our previously published procedure.[18]

N-Fluoren-9-ylmethoxycarbonylaminoacetaldehyde (1)

A mixture of *rac*-3-amino-1,2-propanediol (0.4556 g, 5 mmol), FmocCl (1.2935 g, 5 mmol) and $NaHCO_3$ (0.4201 g, 5 mmol) were dissolved in 1:1 dioxane:H_2O (10 mL). The reaction mixture was stirred at ambient temperature overnight. The solvent was removed by rotary evaporation under reduced pressure. The residue was diluted with 20 mL of water and extracted with dichloromethane (3 × 30 mL) The combined organic extract was dried over magnesium sulfate and evaporated under reduced pressure to give *N*-Fmoc-3-amino-1,2-propanediol as a white solid (1.3935 g, 90%). A mixture of this intermediate (1.2534 g, 4 mmol) and $NaIO_4$ (1.0233 g, 4.8 mmol) were dissolved in 1:1 MeCN:H_2O (8 mL) and allowed to stir at 30°C for 3 hours. The solvents were removed by rotary evaporation and the residue was diluted with 20 mL of water and extracted with dichloromethane (3 × 30 mL). The combined organic extract was dried over magnesium sulfate and evaporated to give the product as a white fluffy solid; yield: 1.1224 g (99%); m.p. 141.0–143.0°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.21 (d $J = 5.0$ Hz, 2H), 4.27 (t $J = 6.8$ Hz, 1H), 4.47 (d $J = 6.8$ Hz, 2H), 5.48 (br m, 1H), 7.36 (m, 2H), 7.45 (m, 2H), 7.64 (d $J = 7.5$ Hz, 2H), 7.81 (d $J = 7.5$ Hz, 2H), 9.71 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 47.1, 51.7, 67.2, 120.0, 125.1, 127.1, 127.8, 141.3, 143.7, 156.3, 196.5.

A Representative Procedure for the Reductive Alkylation

The protected proline derivative **2** (1 mmol) was treated with *p*-toluenesulfonic acid (3 mmol) in MeCN (5 mL). The solvent was removed by

rotary evaporation at 30°C. The residue was dissolved in MeOH (2 mL) followed by successive addition of **1** (1.2 mmol), NaOAc (0.41 g) and $NaBH_3CN$ (1.2 mmol). The solution was stirred at ambient temperature (30°C) for one hour then the solvent was removed by rotary evaporation, diluted with ethyl acetate, and extracted with 10% HCl. The organic layer was washed with aq. $NaHCO_3$ and combined organic phase was dried over magnesium sulfate and evaporated under reduced pressure. The residue was purified by flash column chromatography eluting with hexanes:ethyl acetate (1:1) on silica gel to afford **3**.

A Representative Procedure for Deprotection of the Dpm Ester 3

A mixture of the protected intermediate **3** (0.3–0.5 mmol), anisole (1 mL) and trifluoroacetic acid (3 mL) was stirred at ambient temperature (30°C) overnight. The volatiles were removed under a gentle N_2 stream. Diethyl ether (20 mL) was added to the residue to precipitate the product **4**, which was collected by filtration and dried under vacuum.

Spectroscopic Data of Compounds 3a–3g and 4a–4g

N-2-(N-Fluoren-9-ylmethoxycarbonylamino)ethyl-(4′R)-(N^3-benzoylthymin-1-yl)- (2′R)-proline diphenylmethyl ester (3a)

White foam; yield: 0.6052 g (76%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.87 (s, 3H), 2.01 (m, 1H), 2.65 (m, 1H), 2.78–2.88 (m, 2H), 2.93 (dt, J = 14.4, 9.2 Hz, 1H), 3.18 (m, 1H), 3.31 (m, 1H), 3.39 (m, 1H), 3.49 (m, 1H), 4.26 (t J = 6.8 Hz, 1H), 4.35 (dd J = 9.9, 7.4 Hz, 1H), 4.52 (dd, J = 10.2, 7.3 Hz, 1H), 5.28 (m, 1H), 5.58 (m,1H), 7.03 (s, 1H), 7.32–7.49 (m, 14H), 7.51 (t J = 7.7 Hz, 2H), 7.64–7.73 (m, 3H), 7.82 (d J = 7.4 Hz, 2H), 7.94–8.02 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.7, 36.6, 39.5, 47.3, 52.7, 53.3, 58.2, 65.0, 66.8, 78.2, 111.4, 120.1, 125.3, 127.0, 127.3, 127.9, 128.5, 128.8, 129.3, 130.5, 131.7, 135.2, 138.0, 139.4, 139.5, 141.4, 144.0, 150.0, 156.6, 162.9, 169.5, 172.5; MS (MALDI-TOF) *m/z* 797.64 [M+Na^+]; HRMS (FAB+) *m/z* calcd for $C_{47}H_{42}N_4O_7{\cdot}H^+$: 775.3132, found: 775.3154.

N-2-(N-Fluoren-9-ylmethoxycarbonylamino)ethyl-(4′S)-(N^3-benzoylthymin-1-yl)-(2′S)-proline diphenylmethyl ester (3b)

White foam; yield: 0.6840 g (87%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.87 (s, 3H), 2.01 (m, 1H), 2.66 (m, 1H), 2.77–2.86 (m, 2H), 2.93 (dt, J = 14.9, 9.6 Hz, 1H), 3.18 (m, 1H), 3.31 (d J = 10.7 Hz, 1H), 3.40 (m, 1H), 3.49 (m, 1H), 4.26 (t J = 6.8 Hz, 1H), 4.36 (dd J = 10.1, 7.1 Hz, 1H), 4.53 (dd, J = 10.5, 7.3 Hz, 1H), 5.29 (m, 1H), 5.58 (m,1H), 7.04 (s, 1H), 7.33–7.47 (m, 14H), 7.52 (t J = 7.8 Hz, 2H), 7.64–7.70 (m, 3H), 7.83 (d J = 7.5 Hz, 2H), 7.95–8.00 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.6, 36.6, 39.4, 47.3, 52.5, 53.3, 58.3, 65.0, 66.8, 78.2, 111.5, 120.1, 125.1, 126.9, 127.2, 127.8, 128.4,

128.8, 129.2, 130.5, 131.7, 135.0, 137.6, 139.3, 139.4, 141.4, 144.0, 150.0, 156.6, 162.7, 169.2, 172.5; MS (MALDI-TOF) *m/z* 797.67 [M+Na^+]; HRMS (FAB+) *m/z* calcd for $C_{47}H_{42}N_4O_7 \cdot H^+$: 775.3132, found: 775.3122.

N-2-(N-Fluoren-9-ylmethoxycarbonylamino)ethyl-(4′S)-(N^3-benzoylthymin-1-yl)-(2′R)-proline diphenylmethyl ester (3c)

White foam; yield: 0.3185 g (40%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.06 (s, 3H), 2.25 (m, 1H), 2.53 (m, 1H), 2.72 (m, 2H), 2.96 (m, 1H), 3.27 (m, 2H), 3.40 (m, 1H), 4.04 (dd J = 8.2, 2.8 Hz, 1H), 4.23 (t J = 6.9 Hz, 1H), 4.43 (m, 2H), 5.25 (m, 1H), 5.48 (m, 1H), 6.95 (s, 1H), 7.27–7.46 (m, 15H), 7.49 (t J = 7.9 Hz, 2H), 7.61–7.66 (m,3H), 7.79 (d J = 7.5 Hz, 2H), 7.97 (d J = 7.4 Hz, 2H), ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.6, 35.4, 39.3, 47.3, 51.2, 54.3, 56.0, 63.9, 66.6, 77.6, 111.6, 120.1, 125.1, 127.0, 127.1, 127.8, 128.3, 128.7, 129.3, 130.5, 131.6, 135.1, 137.7, 139.5, 139.6, 141.3, 143.9, 149.7, 156.7, 162.9, 169.3, 171.5; MS (MALDI-TOF) *m/z* 797.68 [M+Na^+]; HRMS (FAB+) *m/z* calcd for $C_{47}H_{42}N_4O_7 \cdot H^+$: 775.3132, found: 775.3144.

N-2-(N-Fluoren-9-ylmethoxycarbonylamino)ethyl-(4′R)-(N^3-benzoylthymin-1-yl)- (2′S)-proline diphenylmethyl ester (3d)

White foam; yield: 0.1652 g (43%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.08 (s, 3H), 2.28 (m, 1H), 2.56 (m, 1H), 2.74 (m, 2H), 2.98 (m, 1H), 3.29 (m, 2H), 3.44 (m, 1H), 4.04 (dd J = 8.2, 2.8 Hz, 1H), 4.24 (t J = 6.7 Hz, 1H), 4.44 (d J = 6.8 Hz, 2H), 5.25 (m, 1H), 6.95 (s, 1H), 7.27–7.44 (m, 14H), 7.51 (t J = 7.8 Hz, 2H), 7.61–7.69 (m,3H), 7.80 (d J = 7.5 Hz, 2H), 7.96 (d J = 7.4 Hz, 2H), ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.6, 35.5, 39.3, 47.3, 51.2, 54.3, 56.0, 63.9, 66.6, 77.6, 111.7, 120.1, 125.0, 127.1, 127.2, 127.8, 128.3, 128.7, 129.3, 130.5, 131.5, 135.2, 137.5, 139.5, 139.6, 141.3, 143.9, 149.7, 156.6, 162.8, 169.2, 171.5; MS (MALDI-TOF) *m/z* 797.80 [M+Na^+]; HRMS (FAB+) *m/z* calcd for $C_{47}H_{42}N_4O_7 \cdot H^+$: 775.3132, found: 775.3138.

N-2-(N-Fluoren-9-ylmethoxycarbonylamino)ethyl-(4′R)-(N^2-isobutyrylguanin-9-yl)-(2′R)-proline diphenylmethyl ester (3e)

White foam; yield: 0.2654 g (69%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.24 (d J = 6.0 Hz, 6H), 2.10–2.22 (m, 1H), 2.55–2.92 (m, 5H), 3.20 (m, 1H), 3.26–3.40 (m, 1H), 3.52 (m, 1H), 4.23 (t J = 6.8 Hz, 1H), 4.35 (d J = 6.8 Hz, 2H), 5.00 (m, 1H), 5.78 and 6.20 (2 × m, 1H), 6.92 (s, 1H), 7.21–7.44 (m, 15H), 7.62 (d J = 7.2 Hz, 2H), 7.75 (d J = 7.2 Hz, 2H), 7.83 and 8.14 (2 × s, 1H), 9.82 and 10.53 (2 × s, 1H), 12.12 and 12.46 (2 × s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 19.1, 19.2, 36.0, 36.9, 39.6, 47.2, 51.9, 53.5, 59.1, 64.7, 66.9, 77.9, 119.9, 120.2, 125.2, 126.9, 127.1, 127.7, 128.3, 128.7, 138.2, 139.4, 141.2, 144.0, 147.9, 148.5, 156.0, 156.8, 172.4, 180.0; MS (MALDI–TOF) *m/z* 766.86 [M+H^+]; HRMS (FAB+) *m/z* calcd for $C_{44}H_{43}N_7O_6 \cdot H^+$: 766.3353, found: 766.3378.

N-2-(N-Fluoren-9-ylmethoxycarbonylamino)ethyl-(4′R)-4′-(N^6-benzoyladenin-9-yl)-(2′R)-proline diphenylmethyl ester (3f)

White foam; yield: 0.3499 g (60%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.25 (m, 1H), 2.80 (m, 1H), 2.89 (m, 1H), 3.02 (m, 2H), 3.16 (m, 1H), 3.39 (m, 1H), 3.47 (m, 1H), 3.62 (m, 1H), 4.29 (t J = 7.2 Hz, 1H), 4.39 (m, 2H), 5.37 (m, 1H), 5.61 (m, 1H), 6.97 (s, 1H), 7.22–7.41 (m, 14H), 7.50 (t J = 7.6 Hz, 2H), 7.58–7.70 (m, 3H), 7.77 (d J = 7.2 Hz, 2H), 8.02 (d J = 7.6 Hz, 2H), 8.65 (s, 1H), 8.82 (s, 1H), 9.36 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 37.2, 39.5, 47.3, 52.1, 53.6, 59.1, 64.5, 66.9, 78.1, 119.9, 122.4, 125.3, 126.9, 127.1, 127.7, 127.9, 128.3, 128.7, 128.8, 132.7, 133.8, 139.3, 141.3, 142.2, 144.1, 149.4, 151.5, 152.4, 156.6, 164.7, 172.5; MS (MALDI–TOF) m/z 784.53 [M+H$^+$]; HRMS (FAB+) m/z calcd for $C_{47}H_{41}N_7O_5{\cdot}H^+$: 784.3247, found: 784.3271.

N-2-(N-Fluoren-9-ylmethoxycarbonylamino)ethyl-(4′R)-(N^4-benzoylcytosin-1-yl)-(2′R)-proline diphenylmethyl ester (3g)

White foam; yield: 0.3231 g (89%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.05 (m, 1H), 2.67 (m, 1H), 2.86 (m, 2H), 2.98 (m, 1H), 3.15 (m, 1H), 3.34 (m, 1H), 3.41 (m, 1H), 3.51 (m, 1H), 4.28 (t J = 7.2 Hz, 1H), 4.42 (t J = 6.8 Hz, 2H), 5.43 (m, 1H), 6.96 (s, 1H), 7.18–7.72 (m, 14H), 7.42 (m, 2H), 7.53 (m, 2H), 7.61–7.72 (m, 2H), 7.79 (d J = 7.2 Hz, 2H), 7.93 (m, 2H), 8.43 (d J = 6.4 Hz, 1H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 36.6, 39.4, 47.2, 53.7, 54.3, 58.6, 65.2, 66.8, 78.1, 97.5, 119.9, 125.3, 127.7, 128.3, 126.9, 127.1, 127.7, 127.8, 128.4, 128.8, 128.9, 133.1, 139.3, 141.3, 144.1, 146.8, 156.0, 156.6, 161.9, 166.5, 172.4; HRMS (FAB+) m/z calcd for $C_{46}H_{41}N_5O_6{\cdot}H^+$: 760.3135, found: 760.3137.

N-2-(N-Fluoren-9-ylmethoxycarbonylamino)ethyl-(4′R)-(thymin-1-yl)-(2′R)-proline (4a)

White solid; yield: 0.2545 g (85%); ^{1}H NMR (400 MHz, DMSO–d_6 + 1 drop TFA) δ 1.77 (s, 3H), 2.35 (m, 1H), 2.83 (m, 1H), 3.20 (m, 1H), 3.37 (m, 2H), 3.51 (m, 1H), 3.69 (m, 1H), 3.94 (m, 1H), 4.26 (t J = 6.8 Hz, 1H), 4.40 (m, 2H), 4.54 (m, 1H), 5.14 (m, 1H), 7.33 (t J = 7.2 Hz, 2H), 7.41 (t J = 7.2 Hz, 2H), 7.57 (s, 1H), 7.69 (d J = 7.2 Hz, 2H), 7.89 (d J = 7.2 Hz, 2H); ^{13}C NMR (100 MHz, DMSO–d_6 + 1 drop TFA) δ 12.7, 34.4, 38.3, 47.2, 53.3, 54.2, 57.5, 66.0, 66.2, 109.7, 120.6, 125.6, 127.6, 128.1, 139.3, 141.2, 144.3, 151.6, 156.7, 164.2, 171.5; MS (MALDI–TOF) m/z 505.13 [M+H$^+$]; HRMS (FAB+) m/z calcd for $C_{27}H_{28}N_4O_6{\cdot}H^+$: 505.2087, found: 505.2102.

N-2-(N-Fluoren-9-ylmethoxycarbonylamino)ethyl-(4′S)-(thymin-1-yl)-(2′S)-proline (4b)

White solid; yield: 0.3711 g (72%); ^{1}H NMR (400 MHz, DMSO–d_6 + 1 drop TFA) δ 1.76 (s, 3H), 2.37 (m, 1H), 2.83 (m, 1H), 3.20 (m, 1H), 3.37 (m, 2H), 3.52 (m, 1H), 3.69 (dd J = 12.5, 10.1 Hz, 1H), 3.95 (dd J = 12.6,

3.9 Hz, 1H), 4.25 (t J = 6.4 Hz, 1H), 4.36–4.44 (m, 2H), 4.54 (m, 1H), 5.15 (m, 1H), 7.32 (t J = 7.3 Hz, 2H), 7.40 (t J = 7.3 Hz, 2H), 7.56 (s, 1H), 7.68 (d J = 7.5 Hz, 2H), 7.87 (d J = 7.5 Hz, 2H); ^{13}C NMR (100 MHz, DMSO–d_6 + 1 drop TFA) δ 12.7, 34.2, 38.1, 47.2, 53.4, 54.3, 57.4, 66.0, 66.3, 109.8, 120.6, 125.6, 127.6, 128.1, 139.2, 141.2, 144.3, 151.6, 156.7, 164.3, 171.1; MS (MALDI–TOF) m/z 505.13 [M+H$^+$]; HRMS (FAB+) m/z calcd for $C_{27}H_{28}N_4O_6{\cdot}H^+$: 505.2087, found: 505.2088.

N-2-(N-Fluoren-9-ylmethoxycarbonylamino)ethyl-(4′S)-(thymin-1-yl)-(2′R)-proline (4c)

White solid; yield: 0.1201 g (72%); ^{1}H NMR (400 MHz, DMSO–d_6 + 1 drop TFA) δ 1.78 (s, 3H), 2.49 (m, 1H), 2.66 (m, 1H), 3.25 (m, 1H), 3.38 (m, 2H), 3.47 (m, 1H), 3.58 (m, 1H), 4.00 (dd J = 11.3, 8.8 Hz, 1H), 4.25 (t J = 6.3 Hz, 1H), 4.40 (d J = 6.6 Hz, 2H), 4.77 (t J = 9.5 Hz, 1H), 4.98 (m, 1H), 7.33 (t J = 7.3 Hz, 2H), 7.42 (t J = 7.4 Hz, 2H), 7.54 (br m, 1H), 7.62 (s, 1H), 7.70 (d J = 7.4 Hz, 2H), 7.90 (d J = 7.5 Hz, 2H); ^{13}C NMR (100 MHz, DMSO–d_6 + 1 drop TFA) δ 12.6, 33.6, 38.6, 47.2, 52.9, 53.0, 55.6, 65.1, 65.9, 110.0, 120.6, 125.6, 127.5, 128.1, 138.8, 141.2, 144.3, 151.3, 156.7, 164.3, 172.3; MS (MALDI–TOF) m/z 505.12 [M+H$^+$]; HRMS (FAB+) m/z calcd for $C_{27}H_{28}N_4O_6{\cdot}H^+$: 505.2087, found: 505.2062.

N-2-(N-Fluoren-9-ylmethoxycarbonylamino)ethyl-(4′R)-(thymin-1-yl)-(2′S)-proline (4d)

White solid; yield: 0.1201 g (72%); ^{1}H NMR (400 MHz, DMSO–d_6 + 1 drop TFA) δ 1.78 (s, 3H), 2.46 (m, 1H), 2.67 (m, 1H), 3.26 (m, 1H), 3.38 (m, 2H), 3.48 (m, 1H), 3.58 (m, 1H), 4.01 (dd J = 10.8, 8.9 Hz, 1H), 4.25 (t J = 6.4 Hz, 1H), 4.40 (d J = 6.7 Hz, 2H), 4.78 (t J = 9.3 Hz, 1H), 4.98 (m, 1H), 7.33 (t J = 7.3 Hz, 2H), 7.41 (t J = 7.4 Hz, 2H), 7.61 (br s, 1H), 7.52 (br m, 1H), 7.69 (d J = 7.3 Hz, 2H), 7.89 (d J = 7.5 Hz, 2H); ^{13}C NMR (100 MHz, DMSO–d_6 + 1 drop TFA) δ 12.6, 33.8, 38.7, 47.2, 52.8, 53.0, 55.7, 65.0, 65.9, 110.0, 120.6, 125.6, 127.5, 128.1, 138.7, 141.2, 144.3, 151.3, 156.7, 164.3, 172.7; MS (MALDI–TOF) m/z 505.00 [M+H$^+$]; HRMS (FAB+) m/z calcd for $C_{27}H_{28}N_4O_6{\cdot}H^+$: 505.2087, found: 505.2063.

N-2-(N-Fluoren-9-ylmethoxycarbonylamino)ethyl-(4′R)-(N^2-isobutyrylguanin-9-yl)-(2′R)-proline (4e)

White solid; yield: 0.1565 g (77%); ^{1}H NMR (400 MHz, DMSO–d_6 + 1 drop TFA) δ 1.14 (d, J = 6.8 Hz, 6H), 2.20 (m, 1H), 2.70 (m, 1H), 2.79 (sept J = 6.8 Hz, 1H), 2.90 (m, 1H), 2.98–3.13 (m, 2H), 3.17–3.29 (m, 2H), 3.50–3.67 (m, 2H), 4.20–4.43 (m, 3H), 5.04 (s, 1H), 7.34 (m, 2H), 7.42 (m, 2H), 7.70 (d J = 7.2 Hz, 2H), 7.89 (d J = 7.2 Hz, 2H), 8.26 (s, 1H), 11.67 (s, 1H), 12.08 (s, 1H); ^{13}C NMR (100 MHz, DMSO–d_6 + 1 drop TFA) δ 19.3, 35.2, 36.2, 39.1, 47.2, 52.1, 54.0, 58.7, 65.4, 65.9, 120.1, 120.6,

125.6, 127.6, 128.1, 138.5, 141.2, 144.4, 148.2, 148.7, 155.4, 156.6, 173.5, 180.5; MS (MALDI–TOF) *m/z* 600.19 [M+H$^+$]; HRMS (FAB+) *m/z* calcd for $C_{31}H_{33}N_7O_6{\cdot}H^+$: 600.2571, found: 600.2568.

N-2-(N-Fluoren-9-ylmethoxycarbonylamino)ethyl-(4′R)-(N^6-benzoyladenin-9-yl)- (2′R)-proline (4f)

White solid; yield: 0.1431 g (75%); ^{1}H NMR (400 MHz, DMSO–d_6 + 1 drop TFA) δ 2.34 (m, 1H), 2.84 (m, 1H), 2.90–3.08 (m, 2H), 3.17–3.39 (m, 3H), 3.75 (m, 1H), 3.88 (m, 1H), 4.21–4.30 (m, 3H), 5.46 (m, 1H), 7.22–7.41 (m, 4H), 7.46 (br m, 1H), 7.57 (t J = 7.6 Hz, 2H), 7.52–7.74 (m, 3H), 7.89 (d J = 7.6 Hz, 2H), 8.08 (d J = 7.6 Hz, 2H), 8.72 (s, 1H), 8.75 (s, 1H); ^{13}C NMR (100 MHz, DMSO–d_6 + 1 drop TFA) δ 35.7, 38.7, 47.1, 52.2, 54.1, 58.4, 65.7, 66.0, 120.6, 125.6, 125.7, 127.6, 128.1, 128.9, 129.0, 133.0, 133.8, 141.2, 143.7, 144.3, 150.7, 151.6, 152.3, 156.7, 166.2, 172.5; MS (MALDI–TOF) *m/z* 618.19 [M+H$^+$]; HRMS (FAB+) *m/z* calcd for $C_{34}H_{31}N_7O_5{\cdot}H^+$: 618.2465, found: 618.2466.

N-2-(N-Fluoren-9-ylmethoxycarbonylamino)ethyl-(4′R)-(N^4-benzoylcytosin-1-yl)- (2′R)-proline (4g)

White solid; yield: 0.2226 g (74%); ^{1}H NMR (400 MHz, DMSO–d_6 + 1 drop TFA) δ 2.47 (m, 1H), 2.98 (m, 1H), 3.24 (m, 1H), 3.41 (m, 2H), 3.57 (m, 1H), 3.77 (m, 1H), 4.14 (m, 1H), 4.28 (m, 1H), 4.36 (m, 2H), 4.60 (m, 1H), 5.13 (m, 1H), 7.32 (m, 2H), 7.42 (m, 2H), 7.54 (m, 2H), 7.58–7.74 (m, 4H), 7.90 (d J = 6.4 Hz, 2H), 8.00 (d J = 7.6 Hz, 2H), 8.20 (d J = 5.6 Hz, 1H); ^{13}C NMR (400 MHz, DMSO–d_6) δ 35.6, 39.0, 47.2, 53.9, 55.8, 58.0, 65.9, 66.0, 96.8, 120.6, 125.6, 127.5, 128.1, 128.9, 129.5, 133.2, 133.7, 141.2, 144.3, 148.6, 155.7, 156.7, 163.2, 167.9, 173.0; MS (MALDI–TOF) *m/z* 594.11 [M+H$^+$]; HRMS (FAB+) *m/z* calcd for $C_{33}H_{31}N_5O_6{\cdot}H^+$: 594.2353, found: 594.2334.

Solid Phase Synthesis of *aep*PNA Oligomers

The Fmoc-protected PNA monomers **4a–4g** were activated just before use as pentafluorophenyl esters by treatment with 1 equiv of pentafluorophenyl trifluoroacetate and DIEA. The products obtained following acid-base extraction to remove DIEA salts were unstable upon prolonged storage and thus were used without further purification. All peptide syntheses were carried out manually in a custom-made glass column with a fritted glass tip (i.d. = 5 mm). The reaction column was filled with TentaGel S RAM Fmoc resin (0.24 meq/g loading, 6.3 mg, 1.5 μmol). The resin, after swollen in DMF, was deprotected by treating with 20% piperidine in DMF (500 μL) for 15 minutes at room temperature with occasional agitation. After washing, the deprotected resin was treated with Fmoc-L-Lys(Boc)-OPfp (9.5 mg, 15 μmol) and HOAt (2.0 mg, 15 μmol) dissolved in anhydrous DMF (35 μL).

After 2 hours at room temperature, the resin was washed exhaustively with DMF and capped with acetic anhydride/DIEA mixture (Ac_2O 10 μL, DIEA 10 μL and DMF 80 μL). Next the Fmoc deprotection was repeated and the resin was treated with an appropriate activated PNA monomer (6.0 μmol) and 7-aza-1-hydroxybenzotriazole (HOAt) (0.8 mg, 6.0 μmol) dissolved in 35 μL DMF for 2 hours. After capping, the deprotection-coupling-capping steps were repeated until the desired sequence was obtained. After final cleavage of the Fmoc protecting group, the PNA was end-capped by acetylation with 10% Ac_2O/DIEA in DMF. With the exception of homothymine sequence, the resin-bound *aep*PNA was treated with 1:1 concentrated aqueous ammonia-dioxane at 60°C overnight to remove the exocyclic amino protecting groups. The crude *aep*PNA was released from the resin by treatment with trifluoroacetic acid (1 mL) at room temperature for 1 hour with occasional agitation. The trifluoroacetic acid was removed by a nitrogen stream (fume hood). The procedure was repeated two more times to ensure a complete cleavage of the peptide from the resin. The crude *aep*PNA was centrifugally washed with diethyl ether (3 times) and was air-dried at room temperature. Analysis and purification was performed by reverse phase HPLC on a Water 600^{TM} system equipped with gradient pump and Water 996 photodiode array detector. A Hypersil C_{18} HPLC column (4.6 × 250 mm, 5 μ particle size) was used for both analytical and preparative purposes. Elution was carried out with a gradient system of 0.1% TFA in acetonitrile/water and monitored at 260 nm. The purity and identity of the products were confirmed by MALDI-TOF mass spectrometry.

T_m Experiments

T_m experiments were performed on a CARY 100 Bio UV-Visible spectrophotometer (Varian Ltd., Australia) equipped with a thermal melt system. The sample for T_m measurement was prepared by mixing calculated amounts of stock oligonucleotide and *aep*PNA solutions together to give final concentration of nucleotides and sodium phosphate buffer (pH 7.0) and the final volumes were adjusted to 3.0 mL by addition of deionized water. The samples were transferred to a 10 mm quartz cell with a Teflon stopper and equilibrated at the starting temperature for 10 minutes. The A_{260} was recorded in steps from 20–90°C (block temperature) with a temperature increment of 1°C/min. The temperature was corrected by applying a linear equation obtained from temperature probe read out. The results were normalized by dividing the absorbance at each temperature by the initial absorbance. T_m values were obtained from first derivative plots.

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