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DNA-, RNA- and self-pairing properties of a pyrrolidinyl peptide nucleic acid with a (2′*R*,4′*S*)-prolyl-(1*S*,2*S*)-2-aminocyclopentanecarboxylic acid backbone

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ABSTRACT

A new pyrrolidinyl peptide nucleic acid (PNA) comprising of an alternate sequence of 4′-nucleobase-modified proline with (2′*R*,4′*S*) configuration and a (1*S*,2*S*)-2-aminocyclopentanecarboxylic acid [(2′*R*,4′*S*)-acpcPNA] backbone was synthesized and its DNA-, RNA- and self-pairing properties studied. T_m and CD studies suggested that the (2′*R*,4′*S*)-acpcPNA forms antiparallel hybrids to DNA and RNA with high sequence and direction specificity. The stability of these hybrids is comparable to those of the (2′*R*,4′*R*)-acpcPNA hybrids previously reported by our group. On the other hand, experiments with a self-complementary sequence indicated that the new (2′*R*,4′*S*)-acpcPNA forms a more stable antiparallel self-hybrid than (2′*R*,4′*R*)-acpcPNA.

A peptide nucleic acid (PNA) is a DNA analogue that binds with single- and double-stranded nucleic acids with high affinity and sequence specificity.[1] In addition to their stronger binding affinity, they are chemically and physiologically more stable than DNA/RNA. These properties lead to a number of applications in the field of diagnostics, therapeutics and biotechnology.[2] After almost two decades since the first peptide nucleic acid (PNA) was introduced by Nielsen et al.[3] this original PNA system (also known as aegPNA) is still perhaps the only PNA system to have found widespread use. Many attempts have been made to improve current PNA technology by incorporating rigidity to the flexible aegPNA backbone in such a way that the PNA backbone is pre-organized for nucleic acid binding.[4,5] Inspired by the concept of foldamers,[6] we recently introduced a series of novel, conformationally rigid PNA analogues with a pyrrolidine core structure in combination with β-amino acid spacers.[7] Preliminary screening based on the (2′*R*,4′*R*) stereoisomer of the nucleobase-modified proline at the 4′ position, which corresponded to the configuration of natural nucleosides, revealed only certain allowable β-amino acid spacers. In particular, the PNA carrying D-aminopyrrolidinecarboxylic acid (DAPC)[7a,b] or (1*S*,2*S*)-2-aminocyclopentanecarboxylic acid (ssACPC)[7c,d] spacers, for example, (2′*R*,4′*R*)-acpcPNA (Scheme 1), can form particularly stable antiparallel hybrids with DNA and less so with RNA.[7d] On the other hand, the effect of changing the configuration of the pyrrolidine in these β-amino acid-containing PNAs has not been systemically investigated.

It is well known that α-anomeric DNA and its backbone-modified analogues can bind to normal β-DNA, albeit with polarity reversal (the two strands must be parallel to each other).[8–10] We therefore proposed that the pyrrolidinyl PNA epimer with a (2′*R*,4′*S*)-prolyl-(1*S*,2*S*)-2-aminocyclopentanecarboxylic acid backbone, that is, (2′*R*,4′*S*)-acpcPNA (Scheme 1), should retain the ability to bind to the target DNA. Furthermore, this epimeric acpcPNA might exhibit different directional specificity (parallel vs antiparallel) and preference for binding to different types of nucleic acid targets (DNA vs RNA) as observed with α-anomeric DNA.[11] Preliminary UV-melting experiments with the remaining three diastereomeric pyrrolidinyl PNAs with the same T_5 sequence and (2′*S*,4′*S*), (2′*S*,4′*R*) and (2′*R*,4′*S*) configurations of the proline moiety revealed that only the (2′*R*,4′*S*) diastereomer binds to poly(dA). To investigate the binding behaviour of this particular epimeric PNA in detail, studies of more complex sequences are required.

The four nucleobase-modified pyrrolidine monomers (A, T, C and G) were prepared starting from the known intermediate **1**[12] according to the method outlined in Scheme 2. In brief, the nucleobases were installed on the pyrrolidine ring, with inversion of

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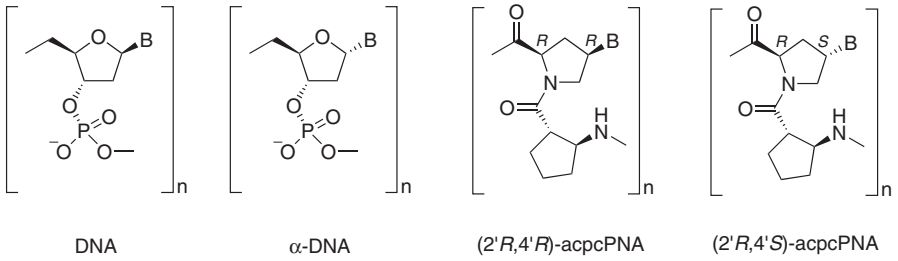
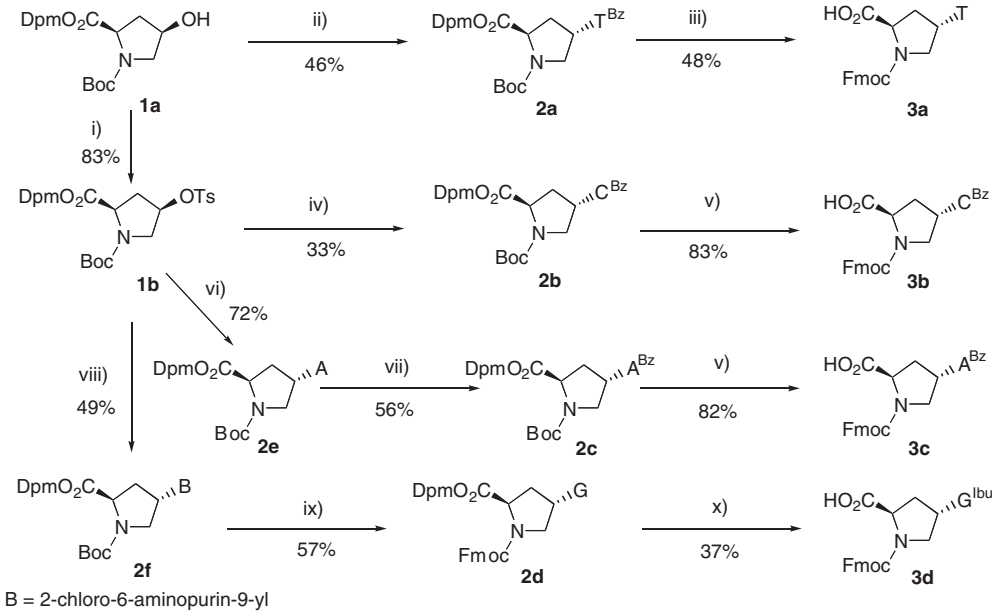


Scheme 1.



i) TsCl, Et_3N, cat. DMAP, CH_2Cl_2, rt, 4 h
ii) N^3-BzT, Ph_3P, DIAD, THF, 0 °C-rt, O/N
iii) a) TsOH, MeCN, rt, 3 h b) FmocCl, DIEA, rt, 1 h c) TFA, anisole, rt, O/N
iv) N^4-BzC, K_2CO_3, DMF, 80 °C, 8 h
v) a) TFA, anisole, rt, 3 h b) FmocOSu, $NaHCO_3$, aq. MeCN, O/N
vi) adenine, K_2CO_3, DMF, 80 °C, 6 h
vii) a) BzCl, pyridine, 0 °C, 2 h b) aq. NH_3, MeOH, rt
viii) 2-amino-6-chloropurine, K_2CO_3, DMF, 80 °C, 2 h
ix) a) TFA, rt, 2 h b) 2M HCl, reflux, 2 h c) FmocOSu, $NaHCO_3$, aq. MeCN, rt, O/N d) Ph_2CHN_2, EtOAc-MeOH, rt, 2 h
x) a) $(CH_3)_2CHCOCl$, pyridine, 0 °C-rt, O/N b) TFA, 1 h

Scheme 2.

configuration, via Misunobu reaction on **1a** (T monomer) or S_N2 substitution of the corresponding tosylate **1b** (C^{Bz}, A^{Bz} and G^{Ibu} monomers; Bz = benzoyl and Ibu = isobutyryl).[12] Due to the poor yield and regioselectivity associated with direct tosyl displacement with A^{Bz} and G^{Ibu}, the displacements were carried out with adenine and 2-amino-6-chloropurine instead. The initial products, **2e** and **2f**, were subsequently transformed into the desired protected monomers by standard nucleoside chemistry. Protecting group exchange from *N*-Boc to *N*-Fmoc afforded the Fmoc-protected monomers **3a–d** for the synthesis of the PNA by Fmoc-SPPS.[13] These monomers, together with Fmoc-(1*S*,2*S*)-2-aminocyclopentanecarboxylic acid[14] were coupled on Tentagel resin equipped with an acid-labile Rink amide linker via their pentafluorophenyl esters,[15] as previously described for similar PNAs.[7,16] One homopyrimidine (**P1**) and two mixed-base PNAs (non self-complementary **P2** and self-complementary **P3**) were synthesized. The identities of all the synthesized PNAs were confirmed by MALDI-TOF mass spectrometry (Table 1, see also Fig. S19–S21, Supplementary data) after cleavage from the resin using trifluoroacetic acid (TFA) and reverse phase HPLC purification.

Table 1
Sequences and characterization data of (2′*R*,4′*S*)-acpcPNA **P1**–**P3**

PNA	Sequence[a] (N → C)	Length (bases)	*m*/*z* (calcd)[b]	*m*/*z* (found)[c]
P1	TTTTTTTT	9	3266.153	3265.575
P2	GTAGATCACT	10	3620.907	3618.854
P3	CGCGAATTCGCG	12	4233.184	4234.538

[a] All PNAs were modified with lysinamide at the C-termini and end-capped by acetylation (**P3**), benzoylation (**P2**) or with lysine (**P1**).
[b] Average mass, calculated for $M{\cdot}H^+$
[c] MALDI-TOF, linear mode, α-cyano-4-hydroxycinnamic acid (CCA) matrix

The interaction of the 9mer homothymine (2′*R*,4′*S*)-acpcPNA **P1** with its complementary DNA (dA_9) was first studied by UV-melting experiments. A single-transition melting curve was observed with a T_m of 67.8 °C.[17] Although this T_m value was ~5 °C lower than

Table 2
*T*m values of hybrids between **P2** and DNA/RNA

Entry	DNA/RNA sequence (5′→3′)	T_m[a] (°C) (2′*R*,4′*R*)	T_m[a] (°C) (2′*R*,4′*S*)	Note
1	dAGTGATCTAC	53.3	50.8	Antiparallel, perfect matched
2	dAGTGCTCTAC	23.8	26.5	Antiparallel, single mismatched
3	dAGTGTTCTAC	29.4	27.4	Antiparallel, single mismatched
4	dAGTGGTCTAC	23.9	28.1	Antiparallel, single mismatched
5	dCATCTAGTGA	<20	<20	Parallel, perfect matched
6	rAGUGAUCUAC	42.3	40.7	Antiparallel, perfect matched
7	rCAUCUAGUGA	<20	<20	Parallel, perfect matched

[a] Conditions: 10 mM sodium phosphate buffer, pH 7.0, 100 mM NaCl; concentration of PNA = DNA = 1 μM, heating rate 1 °C/min. The T_m figures are accurate to within ±0.5 °C.

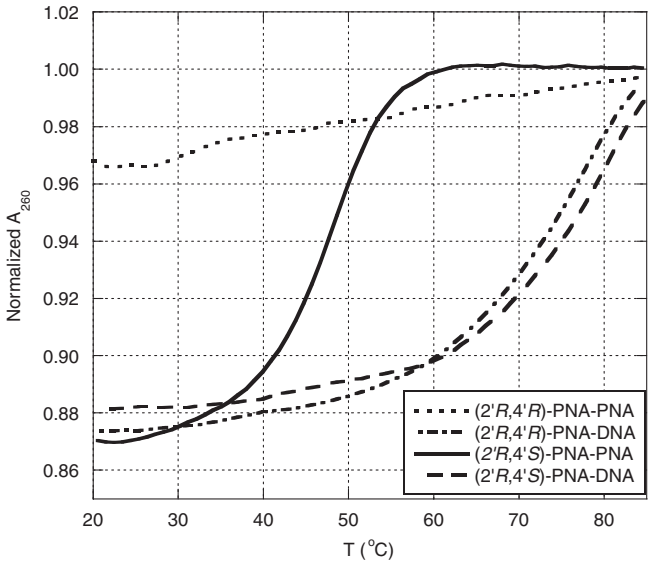


Figure 1. Melting curves of self-complementary PNA P3 in the absence and presence of complementary DNA. The experiments were carried out at a concentration of PNA strand = 2 μM (without DNA target) or 1 μM (with DNA target at 1 μM) in 10 mM sodium phosphate buffer (pH 7.0) containing 100 mM of NaCl, heating rate = 1 °C/min.

that of diastereomeric (2′*R*,4′*R*)-acpcPNA under similar conditions (72.9 °C), we were pleased to find that the (2′*R*,4′*S*)-acpcPNA could indeed retain strong binding to DNA. The T_m values of the hybrids of the mixed-base 10mer (2′*R*,4′*S*)-acpcPNA **P2** and DNA were also measured, and the data compared with (2′*R*,4′*R*)-acpcPNA with the same sequence (Table 2, see also Fig. S22). In this particular sequence, the thermal stability of the complementary antiparallel (2′*R*,4′*S*)-acpcPNA·DNA hybrid was only slightly lower (~2.5 °C) than that of the corresponding (2′*R*,4′*R*)-acpcPNA·DNA hybrid (Table 2, entry 1). No melting could be observed for the corresponding complementary parallel hybrids in both cases, suggesting a strong preference of both PNA diastereomers to bind to their DNA targets in an antiparallel fashion (Table 2, entry 5). When a mismatched base was introduced to the DNA strand, the stabilities of the mismatched PNA–DNA hybrids decreased sharply (Table 2, entries 2–4). The T_m decrease for each (2′*R*,4′*S*)- and (2′*R*,4′*R*)-acpcPNA mismatched hybrid was >20 °C, indicating the high specificity in DNA recognition of both PNA systems. As previously observed with the (2′*R*,4′*R*)-acpcPNA system,[7d] (2′*R*,4′*S*)-PNA also binds with its complementary RNA, selectively, in antiparallel fashion, and with a lower affinity compared to the corresponding DNA hybrids (Table 2, entries 6 and 7).

Finally, the two epimeric PNAs were compared in terms of the ability to self-hybridize. It was established from our previous work that two complementary strands of (2′*R*,4′*R*)-acpcPNA cannot form a hybrid with each other.[7d] The absence of any melting upon heating (2′*R*,4′*R*)-PNA with the same sequence as **P3**, without its DNA target, supported our earlier findings. On the other hand, the self-complementary (2′*R*,4′*S*)-acpcPNA **P3** showed a well-defined melting curve with a T_m value of 47.8 °C suggesting the formation of a PNA·PNA homoduplex (Fig. 1).[18] In the presence of complementary DNA, both PNAs form very stable PNA·DNA heteroduplexes with T_m values of 77.0 °C (2′*R*,4′*R*) and 78.6 °C (2′*R*,4′*S*), respectively. CD spectra (Fig. 2) of the self-complementary PNAs also revealed that the self-complementary (2′*R*,4′*S*)-acpcPNA was more structurally organized than (2′*R*,4′*R*)-acpcPNA with the same sequence, which was consistent with the T_m results. In the presence of the DNA strand, both PNAs become more ordered as shown by the increase in magnitude of the negative CD bands at 208 and 250 nm. The CD spectra also suggested that the helical senses of PNA·DNA hybrids derived from both epimeric PNAs should be similar.

Although only a few other workers have described the syntheses and compared DNA-pairing properties of related diastereomeric pyrrolidinyl PNA systems,[19] these comparisons were limited to homopurine/pyrimidine sequences or chimeric backbones consisting of different type of PNA monomers. The present work is the first example of critical evaluation on mixed sequences with homogeneous, non-chimeric backbones. The exclusive antiparallel binding mode of (2′*R*,4′*S*)-acpcPNA to its complementary DNA is similar to (2′*R*,4′*R*)-acpcPNA,[7d] and is in sharp contrast with the DNA counterparts. In addition, while normal DNA and α-anomeric DNA bind more strongly to RNA than DNA,[11] the corresponding epimeric (2′*R*,4′*S*)-acpcPNA exhibited stronger binding to DNA over RNA being similar to (2′*R*,4′*R*)-acpcPNA.[7d] These, together with the relatively strong, yet highly sequence specific, binding of the new (2′*R*,4′*S*)-acpcPNA to DNA and RNA indicates that the configuration of the proline ring in the pyrrolidinyl PNA can tolerate modification to some extent, without significantly altering the DNA-binding properties. From this study, the only significant difference between the two epimeric PNA systems is that (2′*R*,4′*S*)-acpcPNA can form a relatively stable antiparallel self-complementary hybrid while (2′*R*,4′*R*)-acpcPNA cannot. This emphasizes the delicate effect of stereochemistry on the self-pairing properties of these pyrrolidinyl PNAs. Although the structural basis of this difference is not clear at present, the explanation for the inability of (2′*R*,4′*R*)-acpcPNA to self-hybridize based on steric effects alone may not be sufficient.

This work suggests that the more easily synthesized (2′*R*,4′*S*)-acpcPNA could substitute (2′*R*,4′*R*)-acpcPNA as probes for nucleic acid detection[20] due to their similar DNA- and RNA-binding properties. The unexpected possibility to modulate the self-pairing behaviour of pyrrolidinyl PNA by changing the configuration of the pyrrolidine ring should further expand the potential scope of applications of these pyrrolidinyl PNAs. Although the inability to form a self-pairing hybrid of the original (2′*R*,4′*R*)-acpcPNA system is highly desirable for certain applications such as DNA targeting

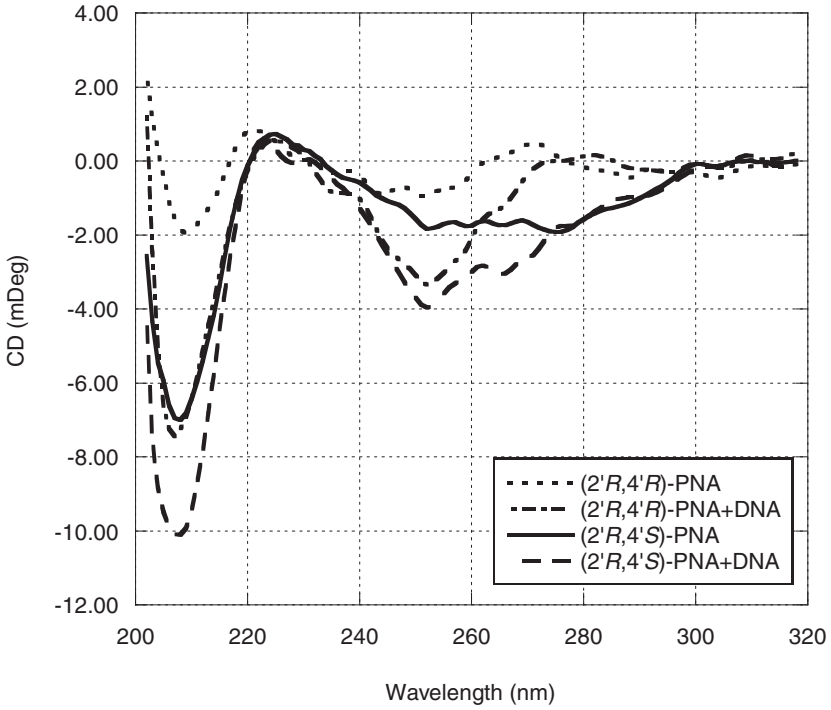


Figure 2. CD spectra of self-complementary (2′*R*,4′*R*)- and (2′*R*,4′*S*)-acpcPNA **P3** in the absence and presence of complementary DNA. The spectra were measured at a fixed concentration of PNA strand = 1 μM and DNA strand = 1 μM in 10 mM sodium phosphate buffer (pH 7.0) containing 100 mM of NaCl at 20 °C.

by double duplex invasion,[21] the self-pairing may also be useful for applications including the construction of PNA-based nanomaterials.[22]

Acknowledgements

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Supplementary data

Supplementary data (experimental details, copies of ^{1}H and ^{13}C NMR spectra of compounds **1b**, **2a–f** and **3a–d**, mass spectra of (2′*R*,4′*R*)-acpcPNA **P1**–**P3** and T_m curves of **P2** hybrids) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2010.08.102.

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13. Spectroscopic data of PNA monomers:
Compound **3a** (T monomer): $[\alpha]_D^{25}$ −2.90 (*c* 1.0, DMF); ^{1}H NMR (400 MHz, DMSO-d_6) (ratio minor/major isomer ~1:1.38) δ_H 1.80 (s, 3H, CH_3 thymine), 2.20–2.32 and 2.58–2.75 [br m, 2H, CH_2(3′) rotamers], 3.44–3.49 and 3.73–3.82 [m, 2H, CH_2(5′) rotamers], 4.18–4.33 (m, 3H, CH Fmoc and CH_2 Fmoc), 4.40–4.43 and 4.53–4.57 [m, 1H, CH(2′) rotamers], 5.03–5.11 [m, 1H, CH(4′)], 7.31–7.35 [m, 3H, CH(6) thymine and CH Ar Fmoc], 7.40–7.45 (m, 2H, CH Ar Fmoc), 7.59 and 7.62 (2 × s, 1H, NH thymine), 7.64–7.66 (m, 2H, CH Ar Fmoc), 7.89–7.91 (m, 2H, CH Ar Fmoc), 11.32 and 11.35 (2 × s, 1H, COOH); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 12.6 (CH_3 thymine), 32.7 and 33.8 [CH_2(3′) rotamers], 47.0 and 47.1 (CH Fmoc), 49.3 and 49.5 [CH(5′) rotamers], 52.3 and 53.3 [CH(4′) rotamers], 57.9 and 58.1 [CH(2′) rotamers], 67.4 and 67.7 (CH_2 Fmoc rotamers), 110.0 [C(5) thymine], 120.6 (CH Ar Fmoc), 125.6 (CH Ar Fmoc), 127.6 (CH Ar Fmoc), 128.2 (CH Ar Fmoc), 138.1 [C(6)H thymine], 141.1 (C Ar Fmoc), 144.1 (C Ar Fmoc), 151.4 [C(2) thymine], 154.0 and 154.3 (CO Fmoc rotamers), 164.2 [C(4) thymine], 173.5 and 173.8 (COOH rotamers); HRMS (ESI) calcd for $C_{25}H_{23}N_3O_6{\cdot}H^+$ 462.1665, found 462.1654.
Compound **3b** (C^{Bz} monomer): $[\alpha]_D^{24}$ −15.1 (*c* 1.0, DMF); ^{1}H NMR (400 MHz, DMSO-d_6) (ratio minor/major isomer ~1:1.37) δ_H 2.34–2.47 and 2.71–2.86 [br m, 2H, CH_2(3′) rotamers], 3.59–3.63 and 3.85–3.94 [m, 2H, CH_2(5′) rotamers], 4.19–4.38 (m, 3H, CH_2 and CH Fmoc rotamers), 4.49 (dd *J* = 8.8, 3.3 Hz) and 4.63 (m) [1H, CH(2′) rotamers], 5.14 [m, 1H, CH(4′)], 7.30–7.38 [m, 3H, CH Ar Fmoc and CH(5) cytosine], 7.33–7.45 (m, 2H, CH Ar Fmoc), 7.51–7.55 (m, 2H, CH Ar Fmoc), 7.62–7.69 (m, 3H, CH Bz), 7.88–7.91 (m, 2H, CH Ar Fmoc), 8.00–8.05 (m, 2H, CH Bz), 8.14 (d *J* = 7.5 Hz) and 8.19 (d *J* = 7.5 Hz) [1H, CH(6) cytosine rotamers], 11.28 (br s, 1H, COOH); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 32.9 and 34.1 [CH_2(3′) rotamers], 47.0 and 47.1 (CH Fmoc rotamers), 50.1 and 50.5 [CH_2(5′) rotamers], 55.4 and 56.4 [CH(4′) rotamers], 57.9 and 58.1 [CH(2′) rotamers], 67.3 and 67.7 (CH_2 Fmoc rotamers), 96.9 [C(5)H cytosine], 120.6 (CH Ar Fmoc), 125.6 (CH Ar Fmoc), 127.6 (CH Ar Fmoc), 128.2 (CH Ar Fmoc), 128.9

(CH Bz), 133.2 (CH Bz), 133.6 (C Bz), 141.1 (C Ar Fmoc), 144.1 (C Ar Fmoc), 147.6 [C(6)H cytosine], 154.1 and 154.2 (CO Fmoc rotamers), 155.4 [C(2) cytosine], 163.2 [C(4) cytosine], 168.0 (CO Bz), 173.5 and 173.8 (COOH rotamers); HRMS (ESI+) calcd for $C_{31}H_{26}N_4O_6{\cdot}H^+$ 551.1931, found 551.1873.

Compound **3c** (A^{Bz} monomer): $[\alpha]_D^{24}$ +7.70 (*c* 1.0, DMF); ^{1}H NMR (400 MHz, DMSO-d_6) (ratio minor/major isomer ~1:1.36) δ_H 2.54–2.68 and 3.01–3.18 [m, 2H, CH_2(3′) rotamers], 3.87–3.98 and 4.03–4.07 [m, 2H, CH_2(5′) rotamers], 4.20–4.38 (m, 3H, CH_2 Fmoc and CH Fmoc rotamers), 4.60 (dd *J* = 8.9, 3.8 Hz) and 4.76 (dd *J* = 9.0, 3.1 Hz) [1H, CH(2′) rotamers], 5.37 [m, 1H, CH(4′)], 7.21–7.45 (m, 4H, CH Ar Fmoc), 7.55–7.70 (m, 5H, CH Ar Fmoc and Bz), 7.82–7.91 (m, 2H, CH Ar Fmoc), 8.06 (m, 2H, CH Bz), 8.59 and 8.62 [2 × s, 1H, CH(8) adenine rotamers], 8.75 and 8.78 [2 × s, 1H, CH(2) adenine rotamers], 11.22 (br s, 1H, COOH); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 33.5 and 34.7 [CH_2(3′) rotamers], 46.5 and 46.6 (CH Fmoc rotamers), 50.2 and 50.6 [CH_2(5′) rotamers], 52.2 and 53.0 [CH(4′) rotamers], 57.5 and 57.7 [CH(2′) rotamers], 66.9 and 67.3 (CH_2 Fmoc rotamers), 120.1 (CH Ar Fmoc), 125.0 and 125.2 (CH Ar Fmoc), 125.6 [C(5) adenine], 127.1 and 127.7 (CH Ar Fmoc), 128.5 (CH Bz), 132.4 (CH Bz), 133.4 (C Bz), 140.6 (C Ar Fmoc), 143.3 and 143.4 [C(8)H adenine rotamers], 143.6 (C Ar Fmoc), 150.3 [C(4) adenine], 151.3 [C(2)H adenine], 152.2 [C(6) adenine], 153.8 (CO Fmoc), 165.8 (CO Bz), 173.0 and 173.3 (COOH rotamers); HRMS (ESI+) calcd for $C_{32}H_{26}N_6O_5{\cdot}H^+$ 575.2043, found 575.2095.

Compound **3d** (G^{Ibu} monomer): $[\alpha]_D^{23}$ −3.43 (*c* 0.5, DMF); ^{1}H NMR (400 MHz, DMSO-d_6) (ratio minor/major isomer ~1:1.32) δ_H 1.13 (d, *J* = 6.0 Hz) and 1.14 (d, *J* = 6.5 Hz) (6H, CH_3 Ibu rotamers), 2.45–2.61 and 2.89–3.04 [m, 2H, CH_2(3′) rotamers], 2.78 (m, 1H, *CH* Ibu), 3.69–3.78 and 3.94–4.02 [m, 2H, CH_2(5′) rotamers], 4.19–4.38 (m, 3H, CH_2 Fmoc and CH Fmoc rotamers), 4.51 (dd *J* = 8.7, 2.9 Hz) and 4.65 (dd *J* = 8.8, 2.2 Hz) [1H, CH(2′) rotamers], 5.06–5.14 [m, 1H, CH(4′)], 7.23–7.45 (m, 4H, CH Ar Fmoc rotamers), 7.59–7.68 (m, 2H, CH Ar Fmoc rotamers), 7.84–7.91 (m, 2H, CH Ar Fmoc rotamers), 8.14 and 8.18 [2 × s, 1H, CH(8) guanine rotamers], 11.73 and 11.76 (2 × s, 1H, NH/OH rotamers), 12.10 and 12.12 (2 × s, 1H, NH/OH rotamers); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C 19.3 (CH_3 Ibu), 34.1 and 35.3 [CH_2(3′) rotamers], 35.2 (CH Ibu), 47.0 and 47.1 (CH Fmoc rotamers), 50.9 and 51.3 [CH_2(5′) rotamers], 51.7 and 52.7 [CH(4′) rotamers], 57.8 and 58.1 [CH(2′) rotamers], 67.3 and 67.7 (CH_2 Fmoc rotamers), 120.6 (CH Ar Fmoc), 120.7 [C(5)H guanine], 125.5 and 125.7 (CH Ar Fmoc rotamers), 127.5 and 127.6 (CH Ar Fmoc rotamers), 128.1 and 128.2 (CH Ar Fmoc rotamers), 138.3 [C(8)H guanine], 141.2 (C Ar Fmoc), 144.1 (C Ar Fmoc), 148.4 and 149.0/149.1 [C(6) and C(2) guanine], 154.0 and 154.2 (CO Fmoc rotamers), 155.3 [C(4) guanine], 173.4 and 173.7 (COOH rotamers), 180.7 (CO Ibu); HRMS (ESI+) calcd for $C_{29}H_{28}N_6O_6{\cdot}H^+$ 557.2149, found 557.2100.

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18. Heating of single-stranded (2′*R*,4′*S*)-acpcPNA with a non-self-complementary sequence such as **P1** resulted in no melting curve. This rules out the possibilities of intramolecular folding or non-specific aggregate formation such as those observed in aegPNA.
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