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Expanding the repertoire of pyrrolidyl PNA analogues for DNA/RNA hybridization selectivity: aminoethylpyrrolidinone PNA (*aepone*-PNA)†

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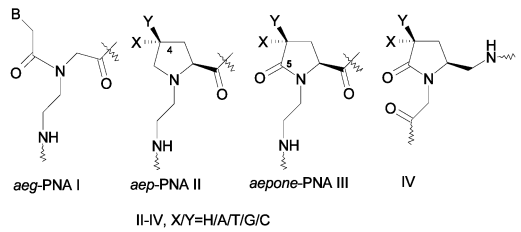
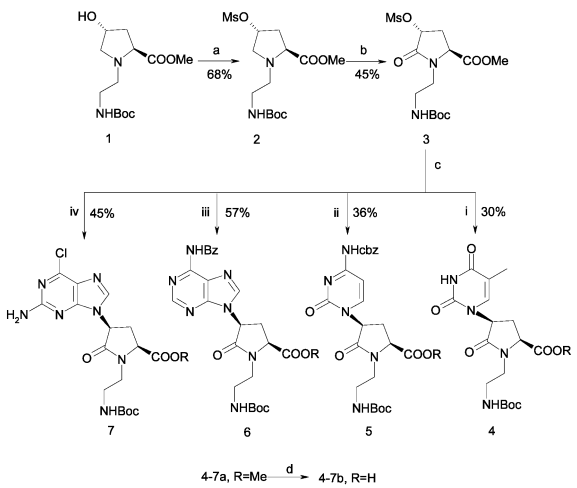
New PNA analogues derived from aminoethylpyrrolidin-5-one backbone show stabilization of *aepone*-PNA:DNA hybrids and destabilization of the corresponding RNA hybrids compared to unmodified PNA.

Peptide Nucleic Acids (PNA, **I**) are one of the most prominent findings of the search for structural analogues of DNA/RNA for antigene/antisense therapeutics.[1] The binding of PNA to the target DNA/RNA sequences occurs with high sequence specificity and this attribute of PNAs for biological and medicinal applications has not yet been fully realized due to poor aqueous solubility, ambiguity in binding orientation specificity (parallel/antiparallel) and lack of sufficient discrimination in binding between target DNA and RNA.[1b,2] Classical PNA is conformationally very flexible and can attain different conformations to accommodate binding to both DNA and RNA. NMR study of PNA oligomers has indicated it to be a complex mixture (up to 2^n) of conformational isomers arising from *cis* and *trans* tertiary amide bonds with a significant barrier to rotational interconversion.[3] PNA hybridization to DNA/RNA is dependent on the tertiary amide conformation and hence affected by the slow rotamer equilibrium. Examination of the crystal structures of PNA/DNA, PNA/RNA, PNA/PNA and PNA_2/DNA triplexes reveal that the linker carbonyl is pointing towards the carboxyl end of PNA.[4]



One way to study the criticality of such structural features is to design structures with frozen rotation of the side chain by locking them into rings as exemplified by the different pyrrolidene based PNA analogues.[5] In one of our earlier modifications, remarkable stabilization of the derived PNA:DNA hybrids was achieved in the chiral and cationic aminoethylprolyl PNA (*aep*-PNA, **II**), having the neutral tertiary amide group of PNA **I** replaced by a protonatable cyclic tertiary amine.[6] In order to avoid the dominance of the non-sequence specific electrostatic component in *aep*-PNA:DNA binding and to get the best characteristics from both the normal PNA and the *aep*-PNA, we resorted to restoring the amide character to the pyrrolidene ring nitrogen. Herein we report the synthesis and evaluation of aminoethylpyrrolidin-5-one PNA (*aepone*-PNA, **III**) having the endocyclic amide CO at C5. The synthesis of all four nucleobase protected monomers (**4,5**) and incorporation of the *aepone*-T monomer **4** into *aeg*-PNA backbone to examine the selectivity in hybridization stability with DNA and RNA is reported. Our present modification with a pyrrolidine-*N*1–CH_2CH_2NH– backbone is quite different from the previously known[7] pyrrolidine-*N*1–CH_2CO backbone (**IV**) as it leads to different C and N termini for the derived PNAs. The chemical synthesis of the target pyrrolidin-5-one system was done *via* selective C5-oxidation of *N*-(aminoethyl-)prolines, wherein despite the available choice of endo- and exo-cyclic N_α–CH_2 groups, the endocyclic 5-CH_2 is preferentially oxidized as confirmed by the reported crystal structure.

The literature methods for the synthesis of 4-substituted pyrrolidin-5-one consist of reaction of proline substrates with RuO_4 generated *in situ* by oxidation of RuO_2 with $NaIO_4$.[8] In these examples, the ring imino nitrogen is protected with a Boc group. In our examples, N1 has an ethylamino substituent that has competing N_α-methylene groups susceptible to oxidation. The reaction of (4*R*)-O-mesyl-N1(ethylamino-N-boc) proline ester **2** with $RuCl_3/NaIO_4$, in a biphasic solvent system, was complete within 1 h and led to a product mixture from which only the major C5-one product **3** could be isolated in 45% yield and successfully characterized (Scheme 1). The identity of the oxidation site in **3** as C5 was unambiguously deduced from its crystal structure (Fig. 1).‡ The oxidation of the C5 endocyclic methylene seems to be preferred over that of exocyclic methylenes, in spite of C5 having an electronegative α-substituent. This compound was used to alkylate N1 of pyrimidines T and C and N9 of purines A and 2-amino-6-chloropurine (precursor for G) which are suitably protected at exocyclic amino groups (Scheme 1) to obtain the (2*S*,4*S*)-*aepone*-PNA monomer esters (**4a–7a**). Upon hydrolysis with LiOH/MeOH esters yielded the monomers (**4b–7b**) suitable for solid phase synthesis of *aepone*-PNA oligomers. It should be pointed out that the synthetic strategy for pyrrolidinone PNA monomers reported here involving prior N-alkylation followed by C5 oxidation is much shorter than the one previously reported[7] for similar analogues.



Scheme 1 Synthesis of aepone-PNA monomers. a) $MeSO_2Cl$, Et_3N in DCM at 0 °C; b) $NaIO_4$, $RuCl_3{\cdot}xH_2O$, CH_3CN–CCl_4–H_2O (1 : 1 : 1.5), 20 min; c) K_2CO_3, 18-crown-6 ether, DMF, 70 °C; i) thymine; ii) N4-cbz-cytosine; iii) N^6-bz-adenine; iv) 2-amino-6-chloropurine.

† Electronic supplementary information (ESI) available: ^1H NMR of **3–7**, mass spectra of **3–7**, **9–11**, HPLC, UV-melting curves and experimental details. See http://www.rsc.org/suppdata/cc/b3/b307362a/

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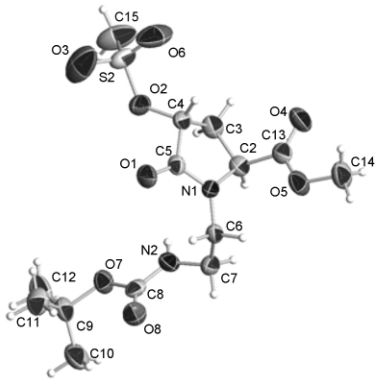

Fig. 1 ORTEP diagram of the crystal structure of **3**.

PNA T_8 oligomers **9–12** incorporating the modified monomers were synthesized using Boc chemistry on β-alanine derivatized Merrifield resin followed by cleavage from the resin with TFA/TFMSA, purification of PNA oligomers by reverse phase HPLC and characterized by MALDI-TOF. The modified *aepone*-T monomer **4b** was incorporated at the C-terminus in PNA **9**, at the C-terminus and centre in PNA **10** and at all positions in PNA **11**. The complementary DNA sequence **13** (GCA_8CG) had GC and CG locks at the 5′- and 3′-ends to avoid slippage of duplexes. The PNA:DNA/RNA complexes were constituted by mixing appropriate strands in a 2 : 1 stoichiometry in buffer followed by heating to 90 °C and annealed by slow cooling to 4 °C to obtain PNA_2:DNA triplexes.

The T_ms of different triplexes as extracted from the derivative plot of temperature dependent UV absorbance (Fig. 2) at 260 nm is shown in Table 1. It is seen that *aepone*-PNA oligomers **9–11** significantly stabilise the derived triplexes with DNA **14** as compared to that from the unmodified PNA oligomer **8** (ΔT_m 16–19 °C) (Fig. 2A). In comparison, the *aepone*-PNAs **9–11** effected destabilization of the triplexes formed with poly(rA), compared to the triplex from unmodified PNA **8** (ΔT_m 12–15 °C) (Fig. 2B). What is significant is that even the completely modified PNA oligomer **11** binds DNA and poly(rA) with a well defined T_m. This result on specificity of hybridization of *aepone*-PNAs **9–11** with preference for significant stabilization of DNA hybrids over RNA hybrids of unmodified PNA **8** is opposite to the selectivity observed for pyrrolidinone-A_8 PNA with opposite polarity;[7] these analogues stabilised RNA hybrids more than the DNA hybrids. The *aepone*-PNA analogues are more akin to the recently reported[9] pyrrolidinyl PNAs in terms of observed selectivities.

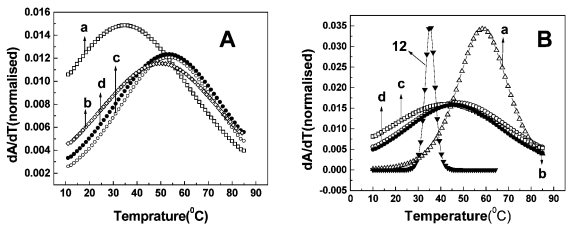

Fig. 2 Derivative UV absorbance (260 nm)–temperature profiles. A) PNA:DNA**13** hybrids and B) PNA:poly(rA) hybrids: a) **8**, b) **9**, c) **10**, d) **11**.

Table 1 UV-T_m (°C) of PNA-DNA/RNA hybrids[a]

Entry	PNA	DNA **13**	poly(rA)
1	**8**	34.8 (14)	58.0 (39)
2	**9**	50.7 (12)	43.1 (19)
3	**10**	50.9 (12)	41.8 (14)
5	**11**	53.3 (10)	45.6 (8)
6	**12**	> 80	35.1

[a] Buffer: 10 mM sodium phosphate, 100 mM NaCl, 0.1 mM EDTA. The values quoted are the average of three experiments and are accurate to ±0.5 °C. Values in parentheses indicate %hyperchromicities.

8. H_2N–T–T–T–T–T–T–T–T-β-ala-COOH (*aeg*-T_8)
9. H_2N–T–T–T–T–T–T–T-**t**-β-ala-COOH
10. H_2N–T–T–T-**t**-T–T–T-**t**-β-ala-COOH
11. H_2N-**t-t-t-t-t-t-t-t**-β-ala-COOH (*aepone*-$\mathbf{t}_8$)
12 H_2N-***t-t-t-t-t-t-t-t***-β-ala-COOH (*aep*-$\boldsymbol{t}_8$)
13 d(GCAAAAAAAACG) (DNA)

The *aep*-PNA oligomer **12** devoid of C5 carbonyl, bound DNA with a very high T_m, melting incompletely even at 80 °C. The strong binding of **12** with DNA is not entirely due to the electrostatic interactions as it showed a lower binding with poly(rA) as compared to PNA **8**. This suggests that the conformational preorganization plays an important role in determining the binding strengths. In this context, the binding pattern of the presently designed *aepone*-PNA is interesting; it has affinity to DNA more than that of PNA, but lower than that of *aep*-PNA and affinity to RNA less than that of PNA and more than that of *aep*-PNA. The tetrahedral nature of pyrrolidine nitrogen in *aep*-PNA is switched back to the planar amide in *aepone*-PNA, as in unmodified PNA with a consequent influence on the backbone conformation. Importantly, the side-chain *syn*/*anti* rotameric equilibrium present in unmodified PNA is not possible in *aepone*-PNA, although the ring nitrogen retains the amide character. Thus *aepone*-PNA (**III**) is an evolved structure by design, combining the features of both PNA (**I**) and *aep*-PNA (**II**). It also emerges from the present data that *aep*-PNA has a selectivity to bind DNA over RNA, and this aspect needs to be confirmed with studies using mixed RNA sequences. The CD spectral features of *aepone*-PNA:DNA/RNA hybrids were similar to that of PNA:DNA/RNA hybrids, suggesting no major differences in base stacking patterns.

In summary, we have reported the synthesis of (2*S*,4*S*)-*aepone*-PNA monomers (**4–7**) as new PNA analogues *via* selective C5 oxidation of *aep*-proline derivatised intermediate **2**. The *aepone*-poly T_8 oligomers (**9–11**) show reverse selectivity in DNA/RNA binding compared with the reported glycylaminomethyl pyrrolidinone[7] and are a useful addition to the growing library of proline/pyrrolidine based PNA analogues[5] to fine tune the binding selectivities. Further studies to delineate the sequence dependent effects of *aepone*-PNA and its stereomers are in progress.

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Notes and references

‡ *Crystal data* for **3**: Crystallised from CH_2Cl_2–MeOH, $C_{14}H_{24}N_2O_8S$, M = 380.41, crystal dimensions 0.61 × 0.09 × 0.05 mm, crystal system: monoclinic, space group $P2_1$, a = 12.739(5), b = 9.294(4), c = 15.994(6) Å, β = 103.419(8)°, V = 1841.9(13) Å^3, Z = 4, D_c = 1.372 g cm^{-3}, μ(Mo-Kα) = 0.219 mm^{-1}, T = 293(2) K, F(000) = 808, max. and min. transmission 0.9885 and 0.8780, 9094 reflections collected, 6134 unique [$I > 2\sigma(I)$], S = 1.109, R value 0.0652, $wR2$ = 0.1213 (all data R = 0.0816, $wR2$ = 0.1283). CCDC 213533. See http://www.rsc.org/suppdata/cc/b3/b307362a/ for crystallographic data in CIF or other electronic format.

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