2-Aminopurine peptide nucleic acids (2-*ap*PNA): intrinsic fluorescent PNA analogues for probing PNA–DNA interaction dynamics

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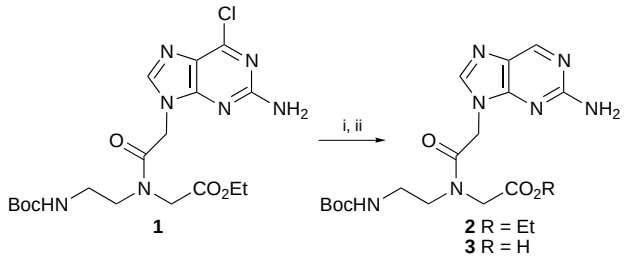
Peptide nucleic acids, containing intrinsically fluorescent 2-aminopurine in place of non-fluorescent adenine, are useful as fluorescent probes to study PNA–DNA interaction dynamics.

Peptide nucleic acids (PNA) are DNA analogues in which the sugar–phosphate backbone is replaced by a peptide amide bond backbone.[1] PNAs exhibit unique properties that set them apart from other traditional DNA analogues. These properties are: (i) unprecedented strong binding to complementary, mixed DNA/RNA sequences to form duplexes, (ii) high specificity in discrimination of mismatched base pairs and (iii) 'strand invasion' at polypurine/pyrimidine stretches on a DNA duplex to form a PNA_2·DNA triplex.[2] This fact, coupled with their resistance to proteases and nucleases and chemical stability over a wide pH range, has led to the development of novel applications that cannot be satisfactorily executed with other DNA analogues. Such applications include blocking and activation of gene expression *via* D-loop complexes, identification of point mutations by PNA-directed PCR clamping, using fluorophore conjugated PNA as probes to locate individual genes in a DNA map and antisense drug development.[2,3]

Most of the structural changes to improve PNA properties so far have centred on backbone modifications to introduce conformational constrain and chirality.[1–4] Herein we report on a novel type of PNA analogue which has intrinsic fluorescence due to the presence of 2-aminopurine (2-*ap*),[5] an isomer of non-fluorescent adenine (6-aminopurine). The base 2-*ap* can be selectively excited in the presence of normal bases, making it an ideal real-time spectroscopic probe to study the dynamics and local environmental changes due to DNA binding with other molecules.[6] Incorporation of this into versatile PNA would therefore add a new dimension to the existing repertoire of PNA properties and potential applications. This is the first report of the synthesis of a PNA analogue which is intrinsically fluorescent, and also examines PNA–DNA complexation *via* fluorescence changes.

The desired PNA monomer **3** carrying a 2-aminopurine moiety was synthesised in two steps (Scheme 1) starting from the previously known compound ethyl *N*-(2-amino-6-chloropurin-9-ylacetyl)-*N*-(2-butoxycarbonylaminoethyl)glycinate **1**.[7] The 6-chloro group in **1** was first removed by hydrogenation over Pd–C to afford **2**, which was followed by hydrolysis of the ester with aq. NaOH to give the corresponding carboxylic acid **3**.‡ This monomer was used directly for incorporation into PNA oligomer **5** without protecting the 2-amino function, since it is inactive for normal peptide coupling conditions. The assembly of the PNA oligomers **4** and **5** was performed manually on Merrifield resin prior functionalised with β-alanine (0.3 equiv. g^{-1}) by standard solid phase peptide synthesis.[7] For the oligomer **5**, the 2-*ap* monomer **3** was used in place of A monomer in the indicated position (*A*). At the end of the synthesis, the oligomers were cleaved from the resin using CF_3SO_3H–TFA and the products assessed by reverse phase HPLC.§ The complementary oligonucleotides **6** and **7** designed for parallel and antiparallel PNA·DNA duplexes were synthesised on an automated DNA synthesiser.



Scheme 1 *Reagents*: i, H_2/Pd–C; ii, aq. NaOH

4 PNA H_2N–T A T T A T T A T T–$CONHCH_2CH_2CO_2H$

5 2-*ap*PNA H_2N–T A T T ***A*** T T A T T–$CONHCH_2CH_2CO_2H$

6 DNA 5' A T A A T A A T A A 3'

7 3' A T A A T A A T A A 5'

8 3' A A A A A A A A A A 5'

A = 2-aminopurine

The fluorescence spectra¶ of the monomer **3** was similar to that of the 2-aminopurine base, with emission at 367 nm upon excitation at 308 nm; the fluorescence intensity changed negligibly with increasing temperature in the range 10–60 °C. The fluorescent observables (λ_{ex}, λ_{em}) from 2-*ap*PNA **5** were similar to those of the monomer, but the fluorescence yield of 2-*ap* in PNA was significantly lower than that of the free base. The PNA oligomer **5** exhibited an increase in intensity as a function of temperature upto 40 °C (total 25%), after which a slight decrease in intensity was observed (Fig. 1). No meaningful changes in λ_{ex}/λ_{em} (±1 nm) were seen with changes in temperature. However, the measurement of fluorescence anisostropy at different temperatures (15 °C, 0.073; 25 °C, 0.060; 35 °C, 0.040; 50 °C, 0.040) showed an appreciable decrease with increasing temperature until *ca*. 40 °C and remained constant thereafter. This behaviour indicates that 2-*ap*PNA, like PNA, is considerably self-organized at low temperatures and melts at higher temperature. Thus, the 2-*ap* residue in **5** senses the local changes in conformation/environment accompanying the self-melting of

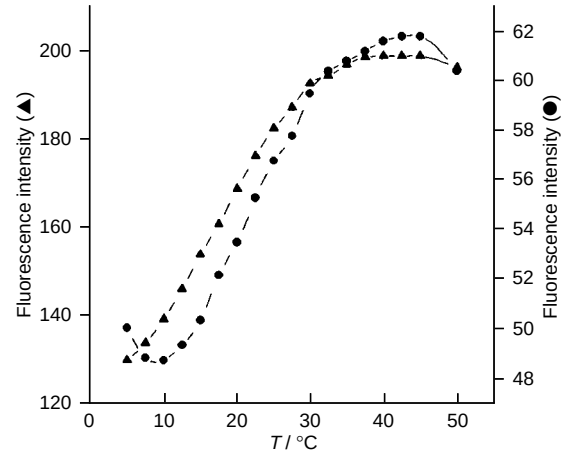


Fig. 1 Fluorescence (λ_{em} 365 nm) melting profiles of (●) 2-*ap*PNA **5** and (▲) 2-*ap*·DNA duplex **5·7**

PNA from an ordered chain to a random coil, a process that also reorients the fluorophore.

The mixed sequence PNAs form both parallel and antiparallel duplexes with appropriate complementary DNA sequences, with antiparallel duplexes being more stable than parallel duplexes.[2] The effect of incorporating 2*ap* in place of A on the thermal stability of duplexes is seen from the UV absorbance–temperature data for PNA·DNA duplexes (T_m; **5·6**, 25.5 °C; **5·7**, 25.0 °C; **4·6**, 28.0 °C; **4·7**, 28.5 °C). The single replacement of A by 2*ap* caused a lowering of T_m by 2–3 °C for both parallel (N/5′, **5·6**) and antiparallel (N/3′, **5·7**) 2*ap*-PNA·DNA duplexes as compared to the corresponding unmodified control duplexes **4·6** and **4·7** respectively. The data indicate a relatively large increase in fluorescence intensity (60%) of 2 *ap* in duplex with temperature as compared to that in 2*ap*PNA alone (25%). Further, a slight blue shift in emission maxima (2 nm) was noticed at 50 °C compared to that at 5 °C, indicating a more hydrophilic environment for 2*ap* after melting. The fluorescence intensity showed a small decrease beyond 40 °C, similar to 2*ap*-PNA (Fig. 1). The parallel PNA·DNA duplex (**5·6**) gave temperature dependent fluorescence spectra (not shown) similar to those of the antiparallel (**5·7**) duplex. Surprisingly, the decrease in fluorescence anisotropy (10%) of the 2*ap* moiety in PNA upon PNA·DNA melting was less than that seen with 2-*ap*PNA alone (45%).

The fluorescence T_m obtained from the data in Fig. 1 for 2*ap*-PNA·DNA melting (**5·7**, 27.5 °C) was less by 5 °C than the T_m for self-melting of 2*ap*-PNA (**5**, 32.5 °C), consistent with the general observation that the thermal stability of the PNA·DNA duplex is lower than that of PNA.[2] It is interesting, but not unprecedented,[6a] to note that the fluorescence T_m of the duplexes (**5·6**, 27.5 °C; **5·7**, 27.0 °C) were *ca*. 2.5 °C higher than the T_m of the corresponding duplexes derived from UV absorbance changes. This difference may arise from the fact that the UV equilibrium melting curves monitor the global duplex dissociation while the fluorescent melting curve monitors local duplex perturbations in the vicinity of the fluorophore.[6] The temperature dependent fluorescence data is also influenced by transient kinetic states that are not reflected in equilibrium data.

The kinetics of the PNA·DNA hybridization process were examined by monitoring the fluorescence emission decay at 367 nm as a function of time after mixing of 2*ap*-PNA **5** with the complementary antiparallel DNA **7** (Fig. 2). The emission intensity decreased exponentially over a period of 6 min [Fig. 2, curve (*c*)] and thereafter remained constant. In contrast, either 2*ap*PNA **5** alone or its mixture with DNA dA_{10}, **8** having three mismatches with PNA, did not exhibit any effect on the PNA fluorescence [Fig. 2, curves (*a*) and (*b*)]. The time-dependent decrease in fluorescence of **5** immediately upon mixing with **7** may therefore be attributed to specific formation of a 2*ap*PNA·DNA hybrid (**5·7**). Similar kinetic behaviour has been previously observed for PNA·PNA hybrid by monitoring the evolution of a circular dichroism signal arising from helical propagation in a 10-mer lys-PNA upon mixing with a complementary PNA sequence[8] where signal saturation occurred at around 6 min. The decay profile observed could be fitted to a double exponential function with time constants of 46 and 290 s, indicating that the fluorescent base 2-*ap* in PNA monitors at least two types of local events, a fast duplex formation by complementary hydrogen bonding followed by a slow reorganization of the helix after duplex formation.

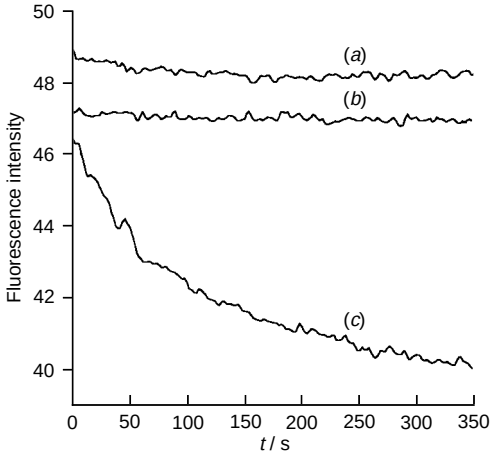


Fig. 2 Fluorescence decay kinetics at 15 °C monitored by emission at 365 nm: (*a*) **5**, (*b*) **5·8** and (*c*) **5·7**

Structural studies of DNA/PNA using fluorescence are best performed without perturbing their structures, which is possible only with the use of intrinsic fluorescence from the base. In this context, 2*ap* is an ideal fluoroprobe since it senses both steady state and dynamic conformational changes. The present results clearly demonstrate that 2-*ap* is easily accommodated in a PNA chain and the intrinsic fluorescence can be employed to monitor PNA self-melting and PNA·DNA duplex transitions. The anisotropy changes suggest that the 2-*ap* residue in a PNA chain is more ordered than its environment in a PNA·DNA duplex. Since 2-*ap* can be placed at any point in a PNA·DNA helix to monitor local events, the type of probes reported here have potential for studying the consequences of backbone modifications on the PNA self-structure and on PNA·DNA duplex. Apart from their utility in diagnostics, these PNA probes may be applicable to other areas, such as the study of cellular uptake and intracellular distribution of PNA by employing fluorescence microscopy, and as sequence specific DNA biosensors.[9]

Footnotes and References

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‡ *Selected data* for **2**: δ_H ($CDCl_3$) 8.65 (s, 1 H, AH8), 7.93–7.90 (s, 1 H, AH6), 5.80 (br s, 1 H, NH), 5.40 (br s, 1 H), 5.00–4.85 (s, 2 H, $glyCH_2$), 4.17–4.05 (s, 2 H, N–CH_2), 4.25 (m, 2 H), 3.67–3.56 (m, 2 H), 3.40–3.32 (m, 2 H), 1.45 (s, 9 H), 1.25 (m, 3 H); δ_C($CDCl_3$) 169.6–169.3 (CO_2Et), 167.3–166.9 (CONH), 159.9 (C2), 156.2 (BocOCON), 153.4 (C8), 148.6 (C6), 143.7 (C4), 126.8 (C5), 79.7 (Me_3CO), 62.1, 61.5, 48.4, 38.6, 28.3 (all CH_2), 13.9 (Me_3C). For **3**: δ_H(D_2O) 8.7 (s, 1 H), 8.33 (s, 1 H), 5.31 and 5.12 (s, 2 H), 4.17 and 4.07 (s, 2 H), 3.7 (t, 2 H), 3.58 (t, 2 H), 3.4 (t, 2 H), 3.23 (t, 2 H), 1.42 and 1.43 (s, 9 H); δ_C($CDCl_3$) 171.6–171.07 (CO_2Et), 167.7–167.2 (CONH), 161.0 (C2), 156.3 (BocOCON), 153.9 (C6), 149.3 (C8), 143.9 (C4), 126.9 (C5), 78.7 (Me_3CO), 50.2, 48.9, 47.6, 43.6, 28.7 (all CH_2), 13.9 (Me_3C).

§ RP 18 column; buffer A: 0.1% TFA in water, buffer B: 0.1% TFA in MeCN gradient 0–40% B in A in 30 min. *R*: **4**, 12.28 min; **5**, 12.26 min.

¶ Fluorescence spectra were recorded on a Perkin-Elmer LS 50B spectrometer, equipped with a flow cell and attached to a Julabo water circulator for variable temperature measurements, using a slit width of 5 nm. The PNA·DNA duplexes were constituted by mixing equimolar amounts of each strand (4 μM) in 10 mM phosphate buffer (pH 7.0, 2 ml) followed by heating at 80 °C for 3 min, cooling to ambient temperature and storage at 4 °C overnight.

1 P. E. Nielsen, *Annu. Rev. Biophys. Biomol. struct.*, 1995, **24**, 167.
2 B. Hyrup and P. E. Nielsen, *Bioorg. Med. Chem. Lett.*, 1996, **4**, 5.
3 D. R. Corey, *Trends Biotechnol.*, 1997, **15**, 224.
4 G. Haaima, A. Lohse, O. Buchardt and P. E. Nielsen, *Angew. Chem., Int. Ed. Engl.*, 1996, **35**, 1939; B. P. Gangamani, V. A. Kumar and K. N. Ganesh, *Tetrahedron*, 1996, **52**, 15 017.
5 D. C. Ward, E. Reich and L. Stryer, *J. Biol. Chem.*, 1969, **244**, 1228.
6 (*a*) D. Xu, K. O. Evans and T. M. Nordlund, *Biochemistry*, 1994, **33**, 9592; (*b*) S. M. Law, R. Eritja, M. F. Goodman and K. J. Breslauer, *Biochemistry*, 1996, **35**, 12 329; (*c*) D. P. Millar, *Curr. Opin. Struct. Biol.*, 1996, **6**, 322; (*d*) R. A. Hochstrasser, T. E. Carver, L. C. Sowers and D. P. Millar, *Biochemistry*, 1994, **33**, 11 971; (*e*) M. W. Frey, L. C. Sowers, D. P. Millar and S. J. Benkovic, *Biochemistry*, 1995, **34**, 9185.
7 P. C. Meltzer, A. Y. Liang and P. Matsudaira, *J. Org. Chem.*, 1995, **60**, 4305.
8 P. Wittung, M. Eriksson, R. Lyang, P. E. Nielsen and B. Norden, *J. Am. Chem. Soc.*, 1995, **117**, 10 167.
9 J. Wang, E. Palecek, P. E. Nielsen, G. Rivas, X. Cai, H. Shiraish, N. Dontha, D. Lere and P. A. M. Farias, *J. Am. Chem. Soc.*, 1996, **118**, 7667.

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