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Nucleic acid binding properties of thyminyl and adeninyl pyrrolidine-amide oligonucleotide mimics (POM)†

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Adeninyl POM was prepared using solid-phase peptide chemistry and shown to exhibit higher affinity for complementary DNA and RNA than the corresponding adeninyl PNA.

There has been considerable interest in modified nucleic acids and oligonucleotide mimics due to their utility as therapeutic[1–3] or diagnostics agents and as tools in molecular biology or target validation.[2–4] Of the many mimics that have been developed to date probably the most widely studied are peptide nucleic acids (PNA).[3,5] Despite success in many applications, PNA suffers from poor aqueous solubility and a tendency to aggregate. In addition, PNA is achiral and can bind in both a parallel and antiparallel orientation with nucleic acids, which can jeopardise sequence specificity. Of late, attention has focused on mimics which contain pyrrolidine-amide backbones.[6,7] Many of these mimics were designed as conformationally restricted chiral PNA analogues[6] and a number including mimics **2**, **3**, and **4** (Fig. 1), exhibit promising properties.

Previously we introduced pyrrolidine-amide oligonucleotide mimics (POM).[7] Unlike the other pyrrolidine containing mimics POM was not designed as a PNA analogue, instead it was intended as a configurational and conformational mimic of natural nucleic acids. Indeed evidence indicates that the (2*R*,4*R*)-pyrrolidine ring in POM adopts a similar conformation to ribose in RNA.[7] In addition, protonation of the pyrrolidine *N*-atom ensures POM possesses excellent water solubility and exhibits electrostatic attraction with oppositely charged nucleic acid targets. It was therefore of little surprise to discover that a T_5-POM, prepared by solution phase synthesis,[7] exhibits very high affinity for both RNA and DNA. Remarkably however the T_5-POM exhibits a very strong kinetic selectivity for RNA over DNA. In this paper we describe the efficient solid-phase synthesis of both thyminyl and adeninyl POM and compare their DNA and RNA binding properties with PNA.

Initially we chose to utilise Fmoc peptide chemistry for the synthesis of oligomers. Accordingly the phthalimido group of **5**[7] was replaced by Fmoc (Scheme 1). Subsequent Boc deprotection followed by *N*-alkylation with *tert*-butyl bromoacetate and then acidolysis gave the thyminyl Fmoc amino acid **8**. This route, which follows on from the earlier synthesis,[7] is not ideal for the other POM monomers (A, C and G) since the base is introduced into the pyrrolidine ring early in the synthesis. A more convergent route to monomer **8** was therefore developed. This involved reduction of the ester **9** followed by mesylation and displacement with azide to give **12**. Acidolysis revealed the amino alcohol **13**, which was *N*-alkylated to provide the *cis*-alcohol **14**. Inversion of the C4-hydroxyl group to give the *trans*-alcohol **16** and introduction of N^3-benzoylthymine using Mitsunobu reactions afforded the (2*R*,4*R*)-pyrrolidine **17**. Reduction and Fmoc protection gave the ester **7**. Surprisingly the N^3-benzoyl protecting group was lost during transformation **17** → **7**. Following this route the adeninyl monomer **21** was also prepared. In this case tosylation of the *trans*-alcohol **16** → **18**, or the *cis*-alcohol **14** → **18**, followed by displacement with N^6-benzoyladenine gave the adeninyl derivative **19**. Azide reduction, Fmoc protection and cleavage of the *tert*-butyl ester gave the required adeninyl Fmoc-amino acid **21**.

Earlier we had prepared a thyminyl POM pentamer with an *N*-terminal phthalimido group (Phth-T_5-POM) by solution phase synthesis.[7] This precluded a direct comparison with PNA, since T_5-PNA requires terminal lysine residues to avoid aggregation and aid aqueous solubility. The thyminyl POM pentamer, LysPOM$(T)_5$-LysNH_2, with *N*- and *C*-terminal lysine residues was therefore prepared on Rink-amide MBHA resin. Coupling reactions involved pre-activation of the T-Fmoc-amino acid **8** (2 equiv.), TBTU (1.9

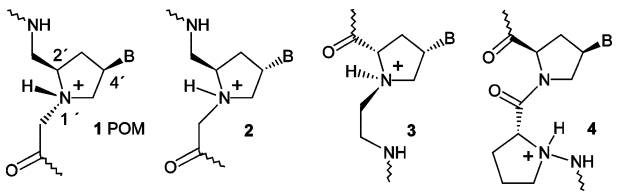
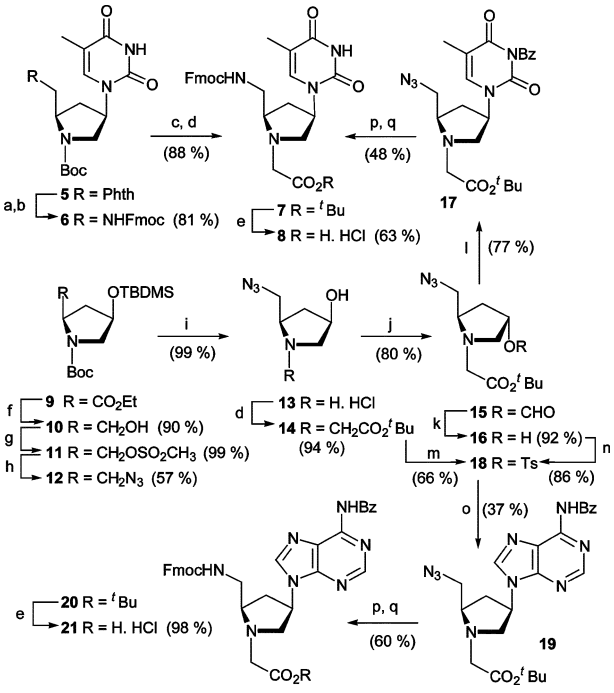


Fig. 1 POM **1**[7] and examples of related PNA analogues.[6]



Scheme 1 a) 40% aq. $MeNH_2$, 50 °C, 2 h; b) Fmoc-Cl, 10% (w/v) aq. Na_2CO_3, dioxane, 0 °C → rt, 18 h; c) 20% CF_3COOH in CH_2Cl_2, rt, 4 h; d) $BrCH_2CO_2tBu$, DIPEA, CH_2Cl_2, 0 °C → rt, 24 h; e) 4 M HCl–dioxane, CH_2Cl_2, rt, 24 h; f) $LiBH_4$, THF, 0 °C 1 h, rt 1 h; g) CH_3SO_2Cl, DIPEA, CH_2Cl_2, 0 °C, 15 min; h) NaN_3, DMF, 80 °C, 16 h; i) 4 M HCl–dioxane, CH_2Cl_2, 0 °C, 15 min, rt, 2 h; j) HCO_2H, PPh_3, DIAD, THF, −30 °C → rt, 18 h; k) conc. aq. NH_3–CH_3OH, 2 h; l) N^3-benzoylthymine, PPh_3, DIAD, THF, −20 °C → rt, 18 h; m) CH_3OTs, PPh_3, DIAD, THF, −10 °C → rt, 18 h; n) TsCl, pyridine, 18 h; o) N^6-benzoyladenine, K_2CO_3, 18-crown-6, DMF, 80 °C, 18 h; p) sat. H_2S in 60% aq. pyridine, 18 h; q) Fmoc-Cl, DIPEA, CH_2Cl_2, 0 °C, 18 h. Bz = benzoyl, Boc = *tert*-butoxycarbonyl, DIAD = diisopropyl azodicarboxylate, DIPEA = diisopropylethylamine, Fmoc = fluoren-9-ylmethoxycarbonyl, Phth = phthalimido, Ts = tosyl.

† This paper is dedicated to Professor Heinz G. Floss on the occasion of his 70th birthday.

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equiv.), HOBT (2 equiv.) and DIPEA (5 equiv.) with an extended coupling time of 4 h. The coupling reactions were monitored by the Kaiser test and coupling efficiencies were calculated to be *ca.* 95% by UV determination of the dibenzofulvene–piperidine adduct after Fmoc deprotection. Cleavage from the resin was achieved using TFA. An adeninyl POM pentamer, LysPOM$(A)_5NH_2$, with an *N*-terminal lysine, was similarly prepared except prior to cleavage from the resin benzoyl deprotection was carried out using 1 : 1 conc. aq. ammonia and dioxane at 55 °C for 16 h. Each oligomer was purified by reverse phase (C8) HPLC to greater than 95% purity and the identity of the products was confirmed by electrospray ionisation mass spectrometry (see ESI).

The DNA and RNA binding properties of the POM 5mers were determined using UV thermal denaturation/renaturation experiments and compared with the corresponding PNA 5mers (Table 1) and DNA 20mers (see ESI). For LysPOM$(T)_5$LysNH$_2$ and poly(rA) an hyperchromic shift of 32% was observed with a denaturation melting temperature (T_m) of 44.8 °C (T_m/base 9.0 °C). Upon renaturation a 28% hypochromic shift was obtained with a T_m of 43.6 °C. The corresponding PNA exhibited slightly higher affinity for poly(rA) (T_m/base 10.8 °C). In contrast $d(T)_{20}$ with poly(rA) exhibits a considerably lower T_m per base of 2.2 °C. Very significant hysteresis was observed in the heating/cooling curves, between LysPOM$(T)_5$LysNH$_2$ and poly(dA), resulting in denaturation and renaturation T_ms that differ by 19.3 °C. Little or no hysteresis was observed in corresponding PNA and $d(T)_{20}$ melting experiments. This indicates that the binding of POM with DNA is much slower than with RNA and is consistent with our previous observations for Phth-T_5-POM.[7] Furthermore this confirms that the unusual kinetic selectivity for RNA over DNA does not depend on the nature of the *N*- or *C*-terminal functionality but is intrinsic to the thyminyl-POM sequence. Notably when poly(dA) and LysPOM$(T)_5$LysNH$_2$ were incubated at room temperature for 48 h prior to UV thermal denaturation the T_m observed was 65 °C (T_m/base = 13 °C), indicating that despite very slow binding POM has very high affinity for the poly(dA). Similar results were also obtained with $r(A)_{20}$ and $d(A)_{20}$ demonstrating that the length of the target nucleic acid strand has little effect on the thermodynamics and kinetics of hybridisation in this case. In addition LysPOM$(T)_5$LysNH$_2$ does not hybridise with non-complementary poly(rC), (rU) and (rG) indicating that base pairing specificity is preserved. Moreover LysPOM$(T)_5$LysNH$_2$ is able to discriminate against a single base pair mismatch as evidenced by the drop in the denaturation T_m with $r(A)_5$ and r(AAGAA) (ΔT_m = −16.3 °C). In contrast, the T_m increases dramatically for LysPNA$(T)_5$LysNH$_2$ with $r(A)_5$ upon insertion of the the single T.G mismatch. This is perhaps due to formation of an atypical complex or aggregate.

Table 1 T_ms for POM and PNA 5mers *vs.* complementary nucleic acids

	T_m/°C[a] (% hypochromic[b]/hyperchromic shift[c])			
	LysPOM$(T)_5$LysNH$_2$		LysPNA$(T)_5$LysNH$_2$	
	cooling	heating	cooling	heating
Poly(rA)	43.6[a] (28)[b]	44.8[a] (32)[c]	53.3 (31)	54.1 (32)
Poly(dA)	25.1 (17)	54.4 (17)	44.2 (25)	48.6 (29)
		65.0[d] (28)[d]		
$r(A)_{20}$	37.2 (31)	40.2 (31)	47.2 (27)	47.9 (28)
$d(A)_{20}$	21.6 (21)	51.6 (24)	35.3 (26)	37.4 (27)
$r(A)_5$	13.6 (14)	34.5 (15)	18.0 (26)	19.5 (28)
r(AAGAA)	14.2 (12)	18.2 (14)	52.8 (7)	60.6 (8)
	LysPOM$(A)_5NH_2$		LysPNA$(A)_5NH_2$	
Poly(rU)	43.4 (35)[b]	50.2 (36)[c]	30.4 (24)	31.0 (25)
Poly(dT)	55.6 (28)	70.3 (31)	47.9 (36)	48.4 (36)
$r(U)_{20}$	38.8 (42)	46.0 (43)	24.1 (25)	24.8 (25)
$d(T)_{20}$	53.0[e] (22)[e]	53.8 (37)	34.6 (35)	34.8 (34)
	31.8[f] (16)[f]			
$r(U)_5$	< 10 (12)	18.3 (11)	n.b.[g]	n.b[g]

[a] All melting experiments were carried out with 42 μM (conc. in bases) of each strand in 10 mM K_2HPO_4, 0.12 M K^+, pH 7.0 (total volume 1.0 cm^3). UV absorbance (A_{260}) was recorded with heating at 5 °C min^{-1} from 23 °C to 93 °C, cooling at 0.2 °C min^{-1} to 15 °C and heating at 0.2 °C min^{-1} to 93 °C. The T_m was determined from the 1st derivative of the slow heating and cooling curve. [b] Hypochromic and [c] hyperchromic shifts are indicated in parentheses and were calculated as follows: [Abs 93 °C − Abs 15 °C] x 100/Abs 93 °C. [d] LysPOM$(T)_5$LysNH$_2$ and poly(dA) were incubated for 48 h before being subjected to slow thermal denaturation (0.2 °C min^{-1}). [e] Probable single strand to duplex and [f] duplex to triplex transitions. [g] No binding (hyperchromicity) was observed.

In addition an efficient solid-phase synthesis of both thyminyl and adeninyl POM from Fmoc amino acids **8** and **21** has been developed. LysPOM$(T)_5$LysNH$_2$ binds with slightly lower affinity than LysPNA$(T)_5$LysNH$_2$ to complementary RNA and DNA, but exhibits a marked kinetic selectivity for RNA over DNA. In contrast LysPOM$(A)_5NH_2$ exhibits considerably higher affinity for complementary RNA and DNA than PNA, with a slight kinetic preference for RNA over DNA. These results corroborate our earlier findings and confirm that POM containing both pyrimidine and purine bases possess excellent nucleic acid binding properties. This combined with the chiral, cationic backbone and excellent aqueous solubility makes POM a potential successor to PNA.

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Notes and references

1 J. Micklefield, *Curr. Med. Chem.*, 2001, **8**, 1157; J. Kurreck, *Eur. J. Biochem.*, 2003, **270**, 1628.
2 M. Petersen and J. Wengel, *Trends Biotechnol.*, 2003, **21**, 74.
3 P. E. Nielsen, *Curr. Opin. Biotechnol.*, 2001, **12**, 16.
4 F. A. Rogers, K. M. Vasquez, M. Egholm and P. M. Glazer, *Proc. Natl. Acad. Sci. USA*, 2002, **99**, 16695; H. Parekh-Olmedo, M. Drury and E. B. Kmeic, *Chem. Biol.*, 2002, **9**, 1073.
5 B. Hyrup and P. E. Nielsen, *Bioorg. Med. Chem.*, 1996, **4**, 5; P. E. Nielsen, *Acc. Chem. Res.*, 1999, **32**, 624.
6 G. Lowe and T. Vilaivan, *J. Chem. Soc., Perkin Trans. 1*, 1997, 539–560; G. Lowe, T. Vilaivan and M. S. Westwell, *Bioorg. Chem.*, 1997, **25**, 321; M. D'Costa, V. Kumar and K. N. Ganesh, *Org. Lett.*, 1999, **1**, 1513; A. Püschl, T. Tedeschi and P. E. Nielsen, *Org. Lett.*, 2000, **2**, 4161; T. Vilaivan, C. Khongdeesameor, P. Harnyuttanakorn, M. S. Westwell and G. Lowe, *Bioorg. Med. Chem. Lett.*, 2000, **10**, 2541; M. D'Costa, V. Kumar and K. N. Ganesh, *Org. Lett.*, 2001, **3**, 1281; V. Kumar, P. S. Pallan, Meena and K. N. Ganesh, *Org. Lett.*, 2001, **3**, 1269; T. Vilaivan and G. Lowe, *J. Am. Chem. Soc.*, 2002, **124**, 9326.
7 D. T. Hickman, P. M. King, M. A. Cooper, J. M. Slater and J. Micklefield, *Chem. Commun.*, 2000, 2251; D. T. Hickman, T. H. S. Tan, J. Morral, P. M. King, M. A. Cooper, J. M. Slater and J. Micklefield, *Org. Biomol. Chem.*, 2003, **1**, 3277.