Stereospecific backbone methylation of pyrrolidine–amide oligonucleotide mimics (POM)†

Anila I. Khan, T. H. Samuel Tan and Jason Micklefield*

Received (in Cambridge, UK) 22nd December 2005, Accepted 30th January 2006
First published as an Advance Article on the web 23rd February 2006
DOI: 10.1039/b518171b

The introduction of (7′*S*)-methyl groups into the backbone of pyrrolidine–amide oligonucleotide mimics (POM) does not interfere with high affinity recognition of complementary nucleic acids, whereas (7′*R*)-methylation disrupts hybridisation significantly.

The redesign of nature's genetic material has resulted in synthetically modified nucleic acids and mimics which have provided us with a deeper understanding of the structure, function and origin of natural DNA and RNA.[1] In addition, nucleic acid mimics continue to be developed as therapeutic agents[2] and are widely used as tools in biomolecular research or as probes in diagnostic and other bioanalytical assays.[3] These molecules also make ideal building materials for the programmed assembly of highly ordered nano-structures and devices, with diverse potential functions.[4] Whilst a large number of modifications and mimics have been introduced remarkably few have been systematically evaluated and shown to possess the pre-requisite properties for hybridisation based applications.[2–4] The need for alternative systems, whose physicochemical properties can be improved and readily fine-tuned, therefore remains acute. To address this need, we introduced the pyrrolidine–amide oligonucleotide mimics (POM) **1** (Fig. 1).[5] POM is a stereochemical and conformational mimic of RNA but is cationic due to protonation of the pyrrolidine ring. It, therefore, possesses excellent water solubility and electrostatic attraction for DNA and RNA. As a result POM hybridises with very high affinity to both complementary nucleic acids, whilst exhibiting unusual kinetic selectivity for RNA over DNA.[5]

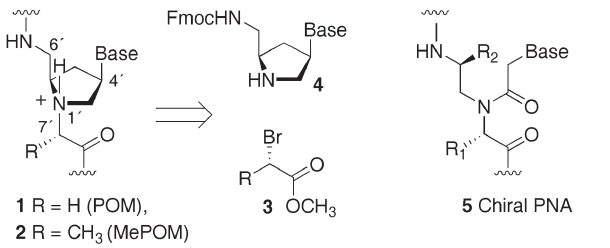


Fig. 1 Structure of POM, (7′*S*)-MePOM and chiral Peptide Nucleic Acids (PNAs). Chiral αPNAs have improved DNA/RNA recognition properties (*e.g.* **5**, $R_1 = (CH_2)_4NH_2$)[7] and improved transport and distribution within cells (**5**, R_1 = carbohydrates or $(CH_2)_3NHC(=NH_2)NH^{2+}$).[8] PNAs substituted with a fluorophore at the γ-position (**5**, R_2 = fluorene) can also be used as molecular beacons.[9a] Base = A, T, C or G.

As we continue to systematically evaluate mixed sequence POMs, we wished to explore the possibility of introducing functionality into the POM backbone that might be used to modulate their physicochemical properties for applications both *in vivo* and *in vitro*.[2,3] Initially, we chose to investigate the effects of introducing substituents at the C7′-position (*e.g.* **2** Fig. 1); we rationalised that these should be synthetically accessible from available homochiral α-bromo esters **3** and pyrrolidines **4**, without deviation from our original synthetic routes.[5] The introduction of substituents into the C7′-positions could affect the backbone torsion angles of POM oligomers as well as the pyrrolidine ring pucker. Moreover these conformational effects are likely to depend on the stereochemistry of the new chiral centres that are generated. For example, it has been reported previously that stereospecific (*S*)-methylation of the C5′-position of amide-linked DNA assists in pre-organising the modified oligonucleotide for more favourable binding with complementary DNA and RNA, whereas (*R*)-methylation has a detrimental effect.[6] Likewise introduction of substituents into the backbone of chiral peptide nucleic acid **5** (Fig. 1) can significantly affect the affinity, selectivity and the orientational preference for binding to nucleic acid targets, depending on the configuration of the new stereogenic centres that are generated.[7]

Provided that C7′-methylation of POM (*e.g.* **2**) does not significantly disrupt the high affinity for binding to nucleic acid targets, then it should be possible to similarly introduce other desirable functional groups using appropriate linkers. For example, the introduction of multiple glycosyl groups, such as *N*-acetylgalactosamine or, alternatively, arginine side chains into the POM backbone might improve cellular uptake and *in vivo* biodistribution and pharmacokinetics.[8] Moreover, the ability to introduce fluorogenic substituents into internal backbone positions, rather than attach them to the nucleobases, is desirable for the design of molecular beacons and other bioanalytical probes.[9]

The synthesis of Fmoc-protected thyminyl MePOM monomers (Scheme 1) was achieved by first removing the phthaloyl group of the thymine derivative **6**[5b] followed by protection of the resulting amine with the Fmoc group to give pyrrolidine **7**. Acidolysis of **7** gave the secondary amine **8**. This was then alkylated with (2*R*)- or (2*S*)-methyl 2-bromopropionate, derived from D- or L-alanine,[10] to give the (7′*S*)- or (7′*R*)-methylated derivatives **9** and **10** respectively. In both cases, a small amount (5–7%) of the opposite diastereoisomer was observed, probably due to racemisation of the

School of Chemistry, The University of Manchester, Faraday Building, Sackville Street, PO Box 88, Manchester, UK M60 1QD.
E-mail: jason.micklefield@manchester.ac.uk

† Electronic supplementary information (ESI) available: Experimental details for MePOM synthesis and selected UV thermal denaturation curves and first derivatives. See DOI: 10.1039/b518171b

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

	T_m/°C[a] Lys-TTTTT-LysNH$_2$					T_m/°C Lys-AAAAA-NH$_2$		
vs.	PNA[5c]	POM[5c]	(7′*R*)-MePOM	(7′*S*)-MePOM	*vs.*	PNA[5c]	POM[5c]	(7′*S*)-MePOM
Poly(rA)	54.1[a]	44.8[a]	29.0[a]	48.0[a]	Poly(rU)	31.0	50.2 (43.4)[c]	50.0
Poly(rA) 0.22 M[b]	51.2	42.2	21.0	44.0	Poly(rU) 1.20 M[b]	36.8	47.8 (41.3)[c]	41.0 (31.0)[c]
Poly(rA) pH 6[b]	57.8	48.6	32.0	44.0	Poly(rU) pH 6[b]	32.2	53.3 (48.4)[c]	52
Poly(rA) pH 8[b]	51.9	39.7	24.0	40.0	Poly(rU) pH 8[b]	29.4	47.2 (38.8)[c]	45.0
Poly(dA)	48.6 (44.2)[c]	65.0 (25.1)[c]	n.b.[d]	n.b.[d]	Poly(dT)	48.4	70.3 (55.6)[c]	68.0 (52.0)[c]
r(A)$_{20}$	47.9	40.2	24.0 (<10.0)	42.0 (32.0)	r(U)$_{20}$	24.8	46.0 (38.8)[c]	37.0 (25.9)[c]
d(A)$_{20}$	37.4	51.6 (21.6)[c]	n.b.[d]	n.b.[d]	d(T)$_{20}$	34.8	53.8 (31.8)[c]	47.0 (45.0)[c]

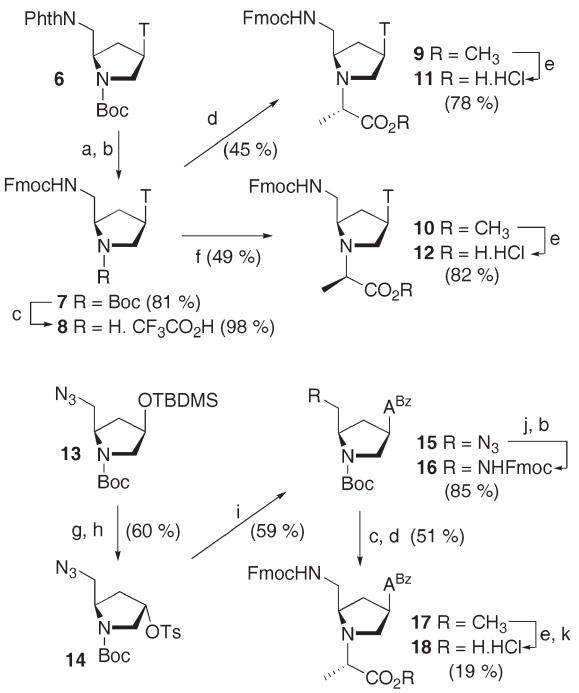
[a] Melting experiments were carried out with 42 μM (conc. in bases) of each strand in 10 mM K_2HPO_4, 0.12 M K^+, pH 7.0 (total volume 1.0 cm^3) unless stated otherwise. UV absorbance (A_{260}) was recorded with heating at 5 °C min^{-1} from 23 °C to 93 °C, cooling at 0.2 °C min^{-1} to 15 °C and heating at 0.2 °C min^{-1} to 93 °C. The T_m was determined from the 1st derivative of the slow heating curve. [b] Ionic strength [K^+] or pH were adjusted accordingly. [c] Where the heating cooling curves were not coincident, indicating relatively slow binding, the T_m values extracted from the cooling curves are shown in brackets. [d] No apparent binding (hyperchromicity) was observed. MePOM 5mers and target DNA/RNA strands do not exhibit hypochromic shifts in the absence of complementary strands under the conditions of these experiments.

bromopropionates. However, these could easily be separated by column chromatography. Hydrolysis of the methyl esters gave the required Fmoc–acids **11** and **12**. These were then used to prepare the Lys-(7′*S*)-MePOM(T)$_5$LysNH$_2$ pentamer and its diastereoisomer Lys-(7′*R*)-MePOM(T)$_5$LysNH$_2$. The lysine residues were included at both the *N*- and *C*-termini to allow direct comparison with PNA and POM Lys(T)$_5$LysNH$_2$ oligomers which were prepared earlier.[5c] Solid phase synthesis was achieved on Rink-amide Novagel resin using Fmoc–peptide chemistry.[5c] The products were then cleaved from the resin using TFA to give *C*-terminal amides which were purified by reverse phase (C18) HPLC and characterised by ESI-MS.

UV thermal denaturation–renaturation experiments were then carried out with both the (7′*S*)- and (7′*R*)-MePOM thyminyl pentamers and the complementary RNA homopolymer poly(rA). This revealed that the (7′*S*)-methyl groups of Lys-(7′*S*)-POM(T)$_5$LysNH$_2$ have a positive effect on binding to RNA resulting in a T_m of 48.0 °C; this is 3.2 °C higher than the original LysPOM(T)$_5$LysNH$_2$ and only slightly lower than the corresponding PNA (Table 1). In contrast, (7′*R*)-methylation had a detrimental effect on the hybridisation with poly(rA), causing a drop in T_m of 15.8 °C compared with the parent POM. A similar trend was also observed for the T_ms with complementary r(A)$_{20}$. However, despite possessing high T_ms with RNA, the Lys-(7′*S*)-POM(T)$_5$LysNH$_2$ exhibited no apparent hybridisation with poly(dA) or d(A)$_{20}$ even after 48 h prior incubation. Previously, we noted that the parent POM binds very slowly to complementary DNA, albeit with high stability.[5] Whether the absence of hybridisation between DNA and the MePOM thyminyl pentamers is due to the instability of the resulting complex, or prohibitively slow kinetics remains to be established.

To further explore the effects of stereospecific methylation of the POM backbone upon hybridisation, the (7′*S*)-methyl adeninyl monomer **18** was prepared from the known azide **13**[5c] (Scheme 1). Desilylation to the corresponding *cis*-alcohol enabled tosylation to be achieved, with inversion of stereochemistry, using methyl tosylate under Mitsunobu conditions.[11] Substitution of the *trans*-tosylate **14** by N^6-benzoyladenine gave the adeninyl derivative **15**. This was transformed through to the methyl ester **17** in an analogous fashion to that described above. However the subsequent hydrolysis of the methyl ester **17** resulted in concomitant cleavage of the benzyl-protecting group, which was thus reinstalled onto the adenine base to give the required monomer **18**. This was then used to prepare Lys-(7′*S*)-MePOM(A)$_5$NH$_2$.

UV melting experiments (Table 1) showed that the Lys-(7′*S*)-MePOM(A)$_5$NH$_2$ exhibited similar affinity for both RNA and DNA as the parent oligomer LysPOM(A)$_5$NH$_2$. Noticeably, the (7′*S*)-MePOM and parent POM adeninyl pentamers both exhibit significantly higher T_ms (*ca.* +15 °C ΔT_m or +3 °C ΔT_m per base) compared with the corresponding LysPNA(A)$_5$NH$_2$. Also, unlike the (7′*S*)-MePOM thyminyl pentamer, the adeninyl pentamer



Scheme 1 Synthesis of MePOM Fmoc-monomers. (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) (2*R*)-methyl 2-bromopropionate,[10] DIPEA, DMF, 0 °C → rt, 18 h; (e) 2 M HCl aq., reflux 18 h; (f) (2*S*)-methyl 2-bromopropionate,[10] DIPEA, DMF, 0 °C → rt, 18 h; (g) TBAF, THF, rt 18 h; (h) CH_3OTs, PPh_3, DIAD, THF, −10 °C → rt, 18 h; (i) N^6-benzoyladenine, K_2CO_3, 18-crown-6, DMF, 80 °C, 18 h; (j) sat. H_2S in 60% aq. pyridine, 18 h; (k) benzyl-Cl, anhydrous pyridine, reflux, 2 h. T = thymin-1-yl, A^{Bz} = N^6-benzoyladenin-9-yl, Phth = phthaloyl.

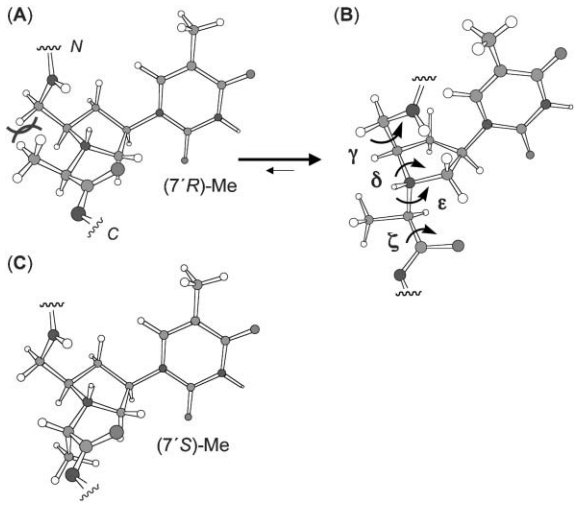


Fig. 2 Idealised helical conformations of MePOMs. (**A**) (7′*R*)-MePOM in an A-type conformation with pyrrolidine ring in the *N*1′-*endo* pucker which is described by the pseudorotation phase angle (*P*) of 45° and δ = 75°.[5b] (**B**) (7′*R*)-MePOM in a B-type, *N*1′-exo conformation (P = 195°, δ = 155°). (**C**) (7′*S*)-MePOM in a A-type, *N*1′-endo conformation (P = 45°, δ = 75°) conformation. In accord with X-ray crystallographic and other data[5b] the γ and ε torsion angles are set at *ca.* 60° and 180° respectively.

binds with extremely high affinity to DNA with a T_m of up to 68.0 °C with poly(dT). Such sequence dependent effects may be due to different modes of hybridisation between the DNA and thyminyl and adeninyl pentamers.[12]

In order to rationalise the general trends observed here, molecular models were generated using torsion angles derived from a recent X-ray crystal structure of a non-methylated POM monomer (see ESI†) and earlier energy minimised structures derived from semi-empirical quantum mechanical calculations, along with NMR data.[5b] All of this structural information is consistent and indicates that the pyrrolidine ring of POM prefers to adopt a *trans* relative configuration about the protonated *N*-atom and most closely resembles the conformation of a typical C3′-*endo* ribose unit in an A-type RNA duplex (Fig. 2). In this idealised conformation, the introduction of a methyl substituent into the C7′-*pro*-*R* position results in a strong 1,3-steric interaction with the C6′-amino methylene substituent. As a result of this, the backbone torsion angle δ is likely to widen to relieve the strain driving the conformation of the pyrrolidine ring from the preferred *N*1′-endo to the next lowest energy *N*1′-exo conformation,[5b] which is more typical of deoxyribose in B-type DNA duplexes. On the other hand, the introduction of a methyl substituent into the C7′-*pro*-*S* position of idealised POM backbone does not result in any major steric interactions. Consequently, the (7′*S*)-MePOM backbone is more likely to remain pre-organised in an A-type helical conformation, which is well established to be most favourable for formation of stable duplexes and triplexes with complementary nucleic acids.[2a]

In summary, we have synthesised (7′*S*)- and (7′*R*)-MePOM oligomers and, using UV thermal denaturation experiments and molecular modelling, we have shown that only (7′*S*)-methylation allows the POM backbone to adopt an optimal conformation for tight binding to complementary nucleic acids. It should therefore be possible to introduce additional functionality, stereospecifically, into the C7′-*pro*-*S* position of the POM backbone without markedly effecting its overall conformation and nucleic recognition properties. As a result of this, we envisage that the physicochemical properties of POMs could be readily modulated according to the type of application for which they might be employed.[2–4,8,9]

This work was supported by the BBSRC (research grant 36/B15998) and by the EPSRC (studentship to A.I.K). We also thank the EPSRC National Mass Spectrometry Centre, University of Wales, at Swansea.

Notes and references

1 (*a*) S. A. Benner, *Science*, 2004, **306**, 625–626; (*b*) A. Eschenmoser, *Science*, 1999, **284**, 2118–2124; (*c*) P. S. Pallan, C. J. Wilds, Z. Wawrzak, R. Krishnamurthy, A. Eschenmoser and M. Egli, *Angew. Chem., Int. Ed.*, 2003, **115**, 5893–5895.

2 (*a*) J. Micklefield, *Curr. Med. Chem.*, 2001, **8**, 1157–1179; (*b*) D. A. Braasch and D. R. Corey, *Biochemistry*, 2002, **41**, 4503–4510; (*c*) T. Shiraishi, S. Pankratova and P. E. Nielsen, *Chem. Biol.*, 2005, **12**, 923–929; (*d*) G. Kolb, S. Reigadas, C. Boiziau, A. van Aerschot, A. Arzumanov, M. J. Gait, P. Herdewijn and J.-J. Toulme, *Biochemistry*, 2005, **44**, 2926–2933.

3 (*a*) B. Vester and J. Wengel, *Biochemistry*, 2004, **43**, 13233–13241; (*b*) P. E. Nielsen, *Curr. Opin. Biotechnol.*, 2001, **12**, 16–20; (*c*) M. Petersen and J. Wengel, *Trends Biotechnol.*, 2003, **21**, 74–81.

4 (*a*) J. Wengel, *Org. Biomol. Chem.*, 2004, **2**, 277–280; (*b*) N. C. Seeman, *Chem. Biol.*, 2003, **10**, 1151–1159; (*c*) P. S. Lukeman, A. C. Mittal and N. C. Seeman, *Chem. Commun.*, 2004, 1694–1695; (*d*) P.-S. Ng and D. E. Bergstrom, *Nano Lett.*, 2005, **5**, 107–111.

5 (*a*) D. T. Hickman, P. M. King, M. A. Cooper, J. M. Slater and J. Micklefield, *Chem. Commun.*, 2000, 2251–2252; (*b*) D. T. Hickman, T. H. S. Tan, J. Morral, P. M. King, M. A. Cooper and J. Micklefield, *Org. Biomol. Chem.*, 2003, **1**, 3277–3292; (*c*) T. H. S. Tan, D. T. Hickman, J. Morral, I. G. Beadham and J. Micklefield, *Chem. Commun.*, 2004, 516–517.

6 A. De Mesmaeker, J. Lebreton, C. Jouanno, V. Fritsch, R. M. Wolf and S. Wendeborn, *Synlett*, 1997, 1287–1290.

7 (*a*) S. Sforza, R. Corradini, S. Ghirardi, A. Dossena and R. Marchelli, *Eur. J. Org. Chem.*, 2000, 2905–2913; (*b*) S. Sforza, G. Haaima, R. Marchelli and P. E. Nielsen, *Eur. J. Org. Chem.*, 1999, 197–204.

8 (*a*) R. Hamzavi, F. Dolle, B. Tavitian, O. Dahl and P. E. Nielsen, *Bioconjugate Chem.*, 2003, **14**, 941–954; (*b*) P. Zhou, M. Wang, L. Du, G. W. Fisher, A. Waggoner and D. H. Ly, *J. Am. Chem. Soc.*, 2003, **125**, 6878–6879.

9 (*a*) E. A. Englund and D. H. Appella, *Org. Lett.*, 2005, **7**, 3465–3467; (*b*) P. J. Hrdlicka, B. Babu, M. D. Sorensen, N. Harrit and J. Wengel, *J. Am. Chem. Soc.*, 2005, **127**, 13293–13299.

10 C. H. Archer, N. R. Thomas and D. Gani, *Tetrahedron: Asymmetry*, 1993, **4**, 1141–1152.

11 M. L. Petersen and R. Vince, *J. Med. Chem.*, 1991, **34**, 2787–2797.

12 The thyminyl and adeninyl POM pentamers, described here and earlier (see ref. 5), do not exhibit hyperchromic shifts in UV melting experiments with non complementary homopolymers, which is consistent with there being no non-specific base pairing. However, the thyminyl POM pentamers could form DNA–POM_2 triplexes in addition to parallel and antiparallel duplexes with poly(dA) and $d(A)_{20}$ (see ref. 5*a*,*b*). Similarly the adeninyl POM pentamers could form parallel and antiparallel duplexes, as well as DNA_2–POM triplexes with both poly(dT) and $d(T)_{20}$. The thermodynamics and kinetics for the formation of these complexes is likely to be significantly different, which may account for the sequence specific effects observed here. The different modes of hybridisation will be thoroughly investigated within our continued investigation in to the synthesis and properties of longer mixed sequence POMs.

