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Synthesis of new chiral PNAs bearing a dipeptide-mimic monomer with two lysine-derived stereogenic centres

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Abstract—The synthesis of new chiral PNA analogues based on lysine is reported. In particular, L- and/or D-lysine-based PNA submonomers bearing two lysine side chains exactly spaced as in the dipeptide Lys-Lys were synthesized and incorporated in the middle of decameric PNA strands, obtaining four diastereomeric (LD, DL, LL and DD) lysine-based chiral PNAs. The hybridization with their complementary antiparallel DNA strand was studied by melting temperature determination and compared with the analogue achiral PNA and chiral PNAs bearing one residue with either of the two lysine enantiomers. The binding abilities were shown to be strongly dependent on the configuration of the stereogenic centres.

1. Introduction

Peptide nucleic acids (PNAs) are a relatively new class of DNA mimics proposed by Nielsen and co-workers in early nineties.[1] The PNA structure is obtained by replacing the charged sugar–phosphate backbone of DNA with a neutral and achiral pseudopeptide backbone based on *N*-(2-aminoethyl)glycine units. PNAs are able to bind the target DNA/RNA sequences through standard Watson–Crick hydrogen bondings with high sequence specificity and affinity.[2] Their outstanding hybridization properties, together with the high chemical and biological stability, have attracted attention from many areas of science, including bioorganic chemistry, drug discovery, molecular biology, diagnostics, prebiotic evolution and also material science.[3] The major limitations which hamper the application of PNAs are ambiguity in the orientation mode of binding (antiparallel vs parallel), solubility not exceeding the low micromolar range in aqueous media and inefficient cellular uptake.[4,5]

Efforts to improve the properties of PNAs by modifying the original backbone have led, among other modifications,[6] to flexible chiral PNA analogues based on different aminoethylamino acids having the stereocentre at the carbon 2 in the backbone (Fig. 1, type I PNA).[7–9] Since chiral PNA synthesis can be affected by racemization, a GC method for detecting the optical purity of PNAs was also developed by our group.[10] A systematic study on the experimental conditions to be used in the coupling reaction on solid phase support were

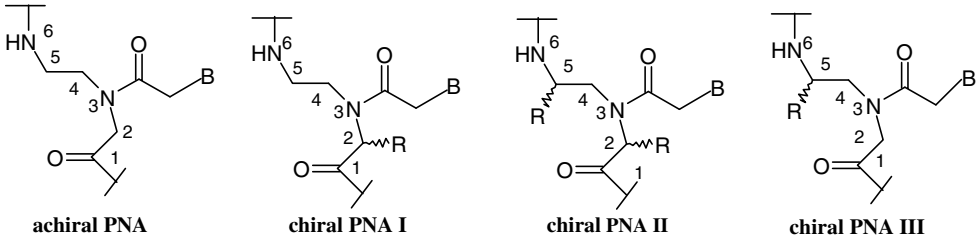

Figure 1. Structure of the chiral PNA of type I, II, III compared to the achiral one. R = amino acid side chain, B = nucleobase.

Keywords: Chiral PNA; Submonomeric strategy; PNA–DNA duplexes.
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recently performed,[11] developing a new 'submonomeric strategy' for the solid phase synthesis of these oligomers:[12] the chiral PNA residue was assembled during the solid phase synthesis, by linking the nucleobase to the aminoethylamino acid backbone directly on the resin. This method allowed for a fast and almost racemization-free synthesis of chiral PNAs.

D-Amino acid-based PNAs of type I favour the DNA binding, in the antiparallel orientation,[13] due to their preferential helix conformation which is right handed, as DNA. Moreover, the use of lysine increases PNA–DNA duplex stability, due to the positive charge on the side chain. A strong chiral constraint in the middle of a PNA strand obtained with three adjacent residues based on D-Lys ('chiral box') highly improved the selectivity in DNA binding.[14] Exploiting this property, 'chiral box' D-Lys-PNA probes were used to detect genomic point mutations with outstanding specificity.[15] Moreover, crystals of a 'chiral box' D-Lys-PNA hybridized to its complementary DNA were also obtained, which were analyzed by means of X-ray diffractometry, providing the very first crystal structure of a PNA–DNA duplex.[16] From these data, the origin of the preferential binding of the D-Lys-PNA of type I was found to be the low intra-strand steric hindrance allowed by the D-configuration of the amino acid. Recently, we have also demonstrated that a L-Lys-'chiral box' PNA of type I shows a preference for the left handed conformation upon antiparallel duplex, thus binding very poorly to a right-handed antiparallel DNA target.[17]

Since the increased chiral constraint in PNAs seemed to be an efficient and quite simple method to improve selectivity, we decided to further follow this line by synthesizing new chiral monomers bearing two, rather than one, lysine-based stereogenic centres (type II PNA, Fig. 1), which have never been reported before in the literature. Beside the expected improvement in binding selectivity, this type of residue more closely mimics a real peptide sequence, since the amino acid side chains will be spaced exactly as in natural oligopeptides. In this way 'truly peptidic' PNAs could eventually be obtained, that is, molecules which not only have the DNA functionality (recognition through nucleobases) but also the protein activity according to the amino acid side chains. In fact, it should be underlined that peptide nucleic acids, despite their name, are essentially used as modified oligonucleotides and that the peptidic behaviour potentially present in the backbone has never actually been exploited.

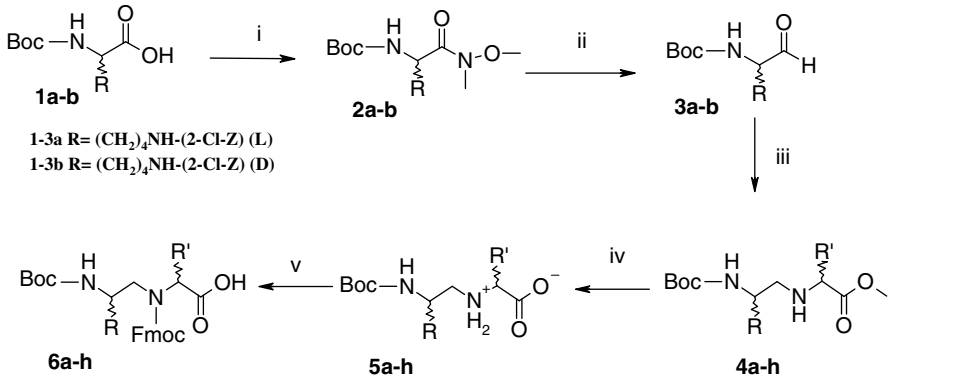
Since every single monomer bears two stereogenic centres, four different diastereomers can be obtained for every residue. In this letter, we report the synthesis of the four diastereomeric two-lysine-submonomers, the synthesis of the chiral decameric PNAs incorporating such residues in the middle of the strand and their preliminary hybridization properties. For comparison, also the synthesis of the submonomers with only one lysine stereogenic centre, in either position 2 (type I PNA, Fig. 1) or position 5 (type III PNA, Fig. 1), their incorporation in the same PNA sequence and the study of their binding properties are also reported. The synthesis of type III (one stereogenic centre in the position 5 of the aminoethyl moiety) monomers has already been reported in the literature,[18,19] though not based on lysine and, not tested as component of a PNA strand.

2. Synthesis of type I, type II and type III PNA submonomeric units

The general structure of the PNA submonomers is reported in Scheme 1 (compounds **6a**–**h**). The rationale of such a structure derives from the submonomeric Boc-based strategy,[12] where an orthogonal Fmoc group is placed onto the secondary amine and subsequently substituted during the solid phase PNA synthesis by the nucleobase.

The synthesis of type I lysine-PNA submonomers (**6a**–**b**), bearing one stereogenic centre in position 2, was done according to the published procedures.[12] The insertion of the lysine in the aminoethyl moiety in position 5 required the previous synthesis of the aldehyde Boc-Lys(2-Cl-Z)-H **3a**–**b**, which was obtained in good yields, starting from the commercially available Boc-Lys(2-Cl-Z)-OH (**1a**–**b**), by reduction with $LiAlH_4$ of the corresponding *N*-methyl-*N*-methoxy amide (**2a**–**b**). The synthesis of type II (stereogenic centres in positions 2 and 5, **6c**–**f**) and type III (stereogenic centre in position 5, **6g**–**h**) submonomers was accomplished essentially in the same way, by performing a reductive amination of Boc-Lys(2-Cl-Z)-H (**3a**–**b**), rather than Boc-aminoacetaldheyde, with lysine methyl ester (in order to obtain **4c**–**f**) or glycine methyl ester (in order to obtain **4g**–**h**) (Scheme 1). Glycine methyl ester is commercially available, whereas lysine methyl ester protected was obtained by esterification of Boc-Lys(2-Cl-Z)-OH **1a**–**b** in strong acidic conditions, which caused a simultaneous Boc group deprotection.[12] The backbones **4a**–**h** were then hydrolized under basic conditions in order to obtain the carboxylic acids **5a**–**h** which were precipitated at their isoelectric point (pH = 5.5) as white powders. The submonomeric units **6a**–**h** were obtained, as previously reported,[12] after alkylation of the secondary amine with Fmoc chloride. The insolubility of **5** and the possibility of racemization were minimized by using the silylating agent bis(trimethylsilyl)-acetamide (BSA), which is known to mask temporarily the carboxylic group, both increasing the solubility and avoiding the reaction with Fmoc chloride which has been demonstrated to lead to racemization.[12] The average yields of steps i–v were 90%, 71%, 48%, 67% and 51%, respectively. In this way, chiral type I PNA submonomers were obtained, together with chiral PNA submonomers of type II and type III (Scheme 1).

All compounds were characterized by ^1H, ^{13}C NMR spectroscopic analyses, mass spectrometry and $[\alpha]_D$. The characterization of the submonomers **6c**–**h** is reported.[20] The optical purity of the compounds **6a**–**b** (type I submonomers) and **6g**–**h** (type III submonomers), bearing only one stereogenic centre, was also evaluated by using the chiral GC method previously developed:[10]



Scheme 1. Reagents and conditions: (i) 0.97 equiv *N*-methyl-*N*-methoxy amine. HCl, 0.95 equiv HBTU, 2 equiv DIEA, DMF. (ii) 4.6 equiv $LiAlH_4$ (1 M in THF), THF. (iii) 1 equiv LysOMe or GlyOMe, 1 equiv $NaBH_3CN$, 1.1 equiv CH_3COOH, CH_3OH. (iv) 10 equiv NaOH $THF/H_2O = 1:1$. (v) 2 equiv BSA, 1.5 equiv DIEA, 2 equiv FmocCl, CH_2Cl_2.

the enantiomeric excess was determined to be $\geqslant 99\%$ both for type I and type III submonomers. These results indicate that the experimental conditions used for each synthetic step do not sensibly affect the configurations of the two stereocentres in positions 2 and 5. The submonomer units based on (2,5)-lysine residues **6c–f** (type II submonomers) are not suitable to GC analysis, but given the optical purity of type I and type III submonomers, it may be deduced that also type II submonomers should be optically pure. This assumption has been confirmed by NMR and HPLC experiments, where no signals due to possible epimerization products have ever been detected.

3. Synthesis of oligomeric PNAs bearing one type I, type II or type III residue

The chosen PNA sequence in which the chiral residues were to be inserted was a decamer already used in many previous studies with chiral PNAs:[7–9,13,14] H-GTAGA**T**-CACT-NH_2. The middle thymine residue (underlined bold) was incorporated as type I, type II or type III residue, obtaining the PNA **7a–h** reported in Table 1.

Incorporation of the achiral monomers was made by standard solid phase synthesis with a Boc strategy on a MBHA resin (loading 0.2 mmol/g) by using an automatic peptide synthesizer.[21] Incorporation of the submonomers to give the chiral thymine PNA residues was done according to the racemization-free submonomeric solid phase strategy, essentially as previously reported:[12] the chiral submonomeric units were inserted by manual coupling with HATU/DIEA protocol and, after Fmoc deprotection, the thyminylacetic acid was introduced by a double coupling with DIC/DhBTOH. Since the presence of the two lysine side chains was thought to induce a high steric hindrance, both the coupling reactions involving the insertion of type II chiral monomers were performed by using more submonomer equivalents (7 equiv vs 5 equiv), more nucleobase equivalents (10 equiv vs 7 equiv) and longer coupling times (1 h vs 30 min) as compared to the previous examples.[12] The success of each coupling reaction was always

Table 1. T_m (°C, calculated as the minimum of the first derivative of the melting curve) obtained by CD at 260 nm of antiparallel PNA–DNA duplexes

PNA type	PNA	T_m PNA/DNA (°C)
I	H-GTAGAT$_{(2L\text{-}Lys)}$CACT-NH_2 (**7a**)	47
I	H-GTAGAT$_{(2D\text{-}Lys)}$CACT-NH_2 (**7b**)	52
II	H-GTAGAT$_{(2L,5L\text{-}Lys)}$CACT-NH_2 (**7c**)	52
II	H-GTAGAT$_{(2D,5D\text{-}Lys)}$CACT-NH_2 (**7d**)	31
II	H-GTAGAT$_{(2D,5L\text{-}Lys)}$CACT-NH_2 (**7e**)	57
II	H-GTAGAT$_{(2L,5D\text{-}Lys)}$CACT-NH_2 (**7f**)	<15
III	H-GTAGAT$_{(5L\text{-}Lys)}$CACT-NH_2 (**7g**)	56
III	H-GTAGAT$_{(5D\text{-}Lys)}$CACT-NH_2 (**7h**)	32
Achiral	H-GTAGATCACT-NH_2	50[13,14]
'L-Chiral box'	H-GTAGA$_{(2L\text{-}Lys)}$T$_{(2L\text{-}Lys)}$C$_{(2L\text{-}Lys)}$ ACT-NH_2	30[17]
'D-Chiral box'	H-GTAGA$_{(2D\text{-}Lys)}$T$_{(2D\text{-}Lys)}$C$_{(2D\text{-}Lys)}$ ACT-NH_2	43[14]

monitored by standard Kaiser and Chloranil qualitative tests. All the PNA oligomers were cleaved from the solid support by treatment with a TFA/TFMSA/*m*-cresol/thioanisole = 6:2:1:1 mixture and obtained, usually quantitatively, after precipitation with diethyl ether. All PNAs (**7a**–**h**) were purified by a semi-preparative RP-HPLC (average yield after purification: 20–30%)[22] and characterized by ESI-MS.[23]

4. Thermal stability of antiparallel PNA–DNA duplexes

As a preliminary study on the effect of the configurations of the stereogenic centres on the DNA binding abilities, all PNA decamers **7a**–**h** were hybridized to the complementary antiparallel DNA sequence (5′-AGTGATCTAC-3′) and the duplex melting temperatures (T_m) were determined by circular dichroism (CD) by measuring at 260 nm the ellipticity variation with the temperature of the solutions containing the duplexes (5 μm) (Table 1).[14] For comparison, also the T_m of the PNA–DNA duplex formed by the corresponding achiral PNA and by the two lysine-'chiral box' PNAs are reported.

From these preliminary experiments it clearly appears that the configuration of the stereogenic centres has a profound influence upon the DNA binding abilities of the PNAs. As it is well known in the literature,[13] when the stereogenic centre is in position 2 (type I) the DNA binding is slightly favoured by the D configuration (**7b** better than **7a**).

It is very interesting to notice that when the stereogenic centre is in position 5 (type III) the situation is reversed and the DNA binding is much more favoured by the L configuration (**7g** much better than **7h**). When the two stereogenic centres are simultaneously present (type II), it is therefore not surprising that the best DNA binding takes place when the stereogenic centre in position 2 has the D configuration and the stereogenic centre in position 5 has the L configuration (**7e**). In this case, the thermal stability of the PNA–DNA duplex is quite outstanding, being 7 °C higher than that achieved by the homologous achiral PNA and 14 °C higher than the D-Lys 'chiral box' PNA. According to our previous results, it seems obvious to speculate that in this case the side chains are very well placed to fit in a right-handed helix. Also the configuration 2L, 5L (**7c**) rather favours DNA binding, suggesting that the influence of the stereogenic centre in position 5 is stronger than that exerted by the stereogenic centre in position 2.

Studies are now in progress in order to define the preferential handedness of the new PNAs bearing two stereogenic centres in the same residue and to confirm whether the stability of the PNA–DNA duplexes can be explained by this preference. Work is in progress to study cases in which the two stereogenic centres induce the same handedness (chiral sinergy) or the opposite handedness (chiral conflict), both for antiparallel and parallel mode of binding. The chiral monomers here reported can be used for the synthesis of PNA, which are simultaneously oligonucleotide and peptide mimics therefore potentially able to show both protein-like and DNA-like recognition properties.

Acknowledgements

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20. Compounds **6c**–**d**: ^{1}H NMR (300 MHZ, DMSO-d_6): δ 7.9–7.8 (m, 2H, aromatic Fmoc), 7.7–7.6 (m, 2H, aromatic Fmoc), 7.4–7.3 (m, 12H, aromatic Fmoc + aromatic 2-Cl-Z), 5.06 (m, 4H, CH_2 2-Cl-Z), 4.3–4.2 (m, 3H, CH + CH_2 Fmoc), 4 (br s, 1H, C(2)-H), 3.7–3.6 (m, 2H, C(4)-H_2), 3.16 (br s, 1H, C(5)-H), 3–2.9 (m, 4H, CH_2 Lys side chain), 1.3–1.1 (m, 12H, CH_2 Lys side chain), 1.33 (s, 9H CH_3 Boc); ^{13}C NMR (75 MHz, DMSO-d_6): δ 175.6, 156.8,

155.9, 155.6, 155.3, 143.9, 143.7, 140.6, 140.5, 134.5, 132.1, 129.4, 129.1, 128.1, 128.7, 127.4, 127.1, 126.9, 125.1, 124.9, 124.8, 76.9, 66.2, 62.3, 55.6, 49.2, 46.6, 40.2, 32.8, 30.5, 29.9, 29.3, 28.1, 24.1, 22.6. ESI (CH_3OH, positive ions) Calculated *m/z*: 941.3 (MNa^+, major isotope), 943.3 (MNa^+, minor isotope); found *m/z*: 941.3 (100%), 943.3 (80%). Compound **6c**: $[\alpha]_D$ −1109 (*c* 1.1, CH_3OH). Compound **6d**: $[\alpha]_D$ +1132 (*c* 1.2, CH_3OH).

Compounds **6e–f**: ^1H NMR (300 MHz, DMSO-d_6): δ 7.9–7.8 (m, 2H, aromatic Fmoc), 7.7–7.6 (m, 2H, aromatic Fmoc), 7.5–7.2 (m, 12H, aromatic Fmoc + aromatic 2-Cl-Z), 5.07 (s, 4H, CH_2 2-Cl-Z), 4.3–4.2 (m, 3H, CH + CH_2 Fmoc), 3.87 (br s, 1H, C(2)-H), 3.53 (br s, 1H, C(5)-H), 3.3–3.2 (m, 2H, C(4)H_2), 3.0–2.9 (m, 4H, CH_2 Lys side chain), 1.4–1.2 (m, 12H, CH_2 Lys side chain), 1.33 (s, 9H, CH_3 Boc); ^{13}C NMR (75 MHz DMSO-d_6): δ 176.1, 157.7, 156.3, 144.1, 143.9, 141.3, 141.1, 134.4, 133.3, 129.6, 129.3, 129.1, 127.7, 127.2, 127.0, 126.7, 125.3, 124.9, 124.5, 79.3, 67.6, 66.7, 64.1, 63.7, 50.3, 49.3, 47.1, 40.8, 33.1, 32.4, 29.4, 29.2, 28.3, 23.5, 22.4. ESI (CH_3OH, positive ions): calculated *m/z*: 941.3 (MNa^+, major isotope), 943.3 (MNa^+, minor isotope); found *m/z*: 941.4 (100%), 943.4 (80%). Compound **6e**: $[\alpha]_D$ +13.1 (*c* 1.3 in CH_3OH). Compound **6f**: $[\alpha]_D$ −12.7 (*c* 1.0 in CH_3OH).

Compounds **6g–h**: ^1H NMR (300 MHz, DMSO-d_6): δ 7.9–7.8 (m, 2H, aromatic Fmoc), 7.7–7.6 (m, 2H, aromatic Fmoc), 7.5–7.3 (m, 12H, aromatic Fmoc + aromatic 2-Cl-Z), 5.07 (s, 2H, CH_2 2-Cl-Z), 4.4–4.2 (m, 3H, CH + CH_2 Fmoc), 3.8–3.4 (m, 7H, C(5)-H + C(2)H_2 + CH_2 Lys side chain), 1.5–1.1 (m, 6H, CH_2 Lys side chain), 1.34 (s, 9H, CH_3 Boc); ^{13}C NMR (75 MHz, DMSO-d_6, some signals splitted, major rotamer only reported): δ 172.9, 156.3, 156.0, 155.5, 144.4, 144.0, 141.0, 140.8, 134.9, 132.5, 129.8, 129.5, 129.2, 127.8, 127.5, 125.7, 125.3, 77.5, 67.1, 66.8, 62.7, 51.3, 49.3, 46.9, 40.0, 31.8, 29.5, 28.5, 23.1. ESI (CH_3OH, positive ions): calculated *m/z*: 702.3 (MNa^+, major isotope), 704.3 (MNa^+, minor isotope); found *m/z*: 702.5 (100%), 704.5 (40%). Compound **6g**: $[\alpha]_D$ +6.1 (*c* 1.2 in CH_3OH). Compound **6h**: $[\alpha]_D$ −6.5 (*c* 1.1 in CH_3OH).

21. Synthesis was performed on a ABI433A peptide synthesizer following the standard procedures indicated by the company.
22. Semipreparative HPLC conditions: column C18 (250 × 10 mm, 5 μm, 300 Å); eluents: A:100% H_2O + 0.1% TFA, B: 60% H_2O/40% CH_3CN + 0.1% TFA; gradient: from 100% A to 100% B in 35 min; flow rate: 4 ml/min; detector: UV $\lambda = 260$ nm.
23. PNA **7a–b** ESI-MS (H_2O, positive ions): calculated *m/z*: 934.0 $[MH_3]^{3+}$, 700.7 $[MH_4]^{4+}$, 560.8 $[MH_5]^{5+}$; found *m/z*: 934.1, 700.8, 561.1. PNA **7c–d** ESI-MS (H_2O, positive ions): calculated *m/z*: 957.3 $[MH_3]^{3+}$, 718.2 $[MH_4]^{4+}$, 574.8 $[MH_5]^{5+}$, 479.1 $[MH_6]^{+6}$; found *m/z*: 957.2, 718.3, 574.9, 479.3. PNA **7e–f** ESI-MS (H_2O positive ions): calculated *m/z*: 957.3 $[MH_3]^{3+}$, 718.2 $[MH_4]^{4+}$, 574.8 $[MH_5]^{5+}$, 479.1 $[MH_6]^{+6}$; found *m/z*: 957.3, 718.3, 574.9 479.2. PNA **7g–h** ESI-MS (H_2O positive ions): calculated *m/z*: 1400.5 $[MH_2]^{+2}$, 934.0 $[MH_3]^{3+}$, 700.7 $[MH_4]^{4+}$, 560.8 $[MH_5]^{5+}$, 467.5 $[MH_5]^{5+}$; found *m/z*: 1400.2, 934, 700.9, 560.9, 467.9.