Fast, Solid-Phase Synthesis of Chiral Peptide Nucleic Acids with a High Optical Purity by a Submonomeric Strategy

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Keywords: Peptide nucleic acids / Chirality / Solid-phase synthesis / Optical purity / Submonomeric strategy

The solid-phase synthesis of chiral peptide nucleic acids (PNAs) usually results in partial epimerization of the products, since the α-nitrogen atom of the amino acid is involved in an amidic bond. It is also time-consuming, since all the chiral monomers bearing different nucleobases have to be independently synthesized. In order to prevent racemization and to speed up the synthetic procedure we adopted a submonomeric approach by using a solid-phase, Boc-based PNA synthesis in which the chiral backbone orthogonally N^{α}-Fmoc-protected (submonomer) was first linked to the growing chain on the resin, followed by Fmoc-deprotection and derivatization with the carboxymethylnucleobase. The submonomer bearing the D-lysine residue was designed by protecting the N^{α}-(aminoethyl)amino acid moiety with an Fmoc protecting group, compatible with standard Boc chemistry, and with the use of an MBHA-PS resin, normally employed for PNA synthesis. Different synthetic pathways towards the desired submonomer were studied by using the amino acid D-lysine as a chiral synthon, obtaining a fast method leading to a high yield and an excellent enantiomeric excess of the submonomer. The solid-phase submonomeric reaction conditions were optimized for the synthesis of a thyminyl PNA dimer and then used to synthesize two different chiral PNAs. In this way two advantages were obtained: a lower degree of racemization in the coupling step during the solid-phase synthesis and the possibility of using the same submonomer for every different nucleobase. All the D-lysine-based chiral PNAs were obtained in good yields and, as compared with PNAs synthesized by other coupling methods, showed the highest optical purity reported so far.

Introduction

Peptide nucleic acids (PNAs) are pseudopeptide DNA mimics, first proposed by Nielsen and co-workers in 1991,[1] which have attracted much interest because of their high specificity and affinity for DNA.[2] Due to their outstanding behavior, PNAs are currently used in a plethora of biological applications which require specific DNA recognition, such as point mutation recognition,[3] genetic probing by in situ hybridisation,[4,5] PCR clamping,[6] transcription inhibition,[7] translation inhibition,[8] and for both diagnostic and therapeutic purposes.[9]

Since their discovery many modifications of the original PNA monomeric units have been reported in order to improve the binding stability to the complementary nucleic acids and/or the specificity of complexation.[10,11] Among the proposed modifications, one of the most intriguing is the introduction of a stereogenic centre at the α-carbon atom of the (aminoethyl)glycine unit, which result in chiral PNAs, such as those depicted in Figure 1.

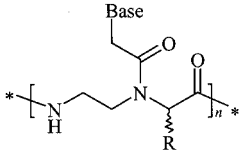

Figure 1. Chiral PNA based on amino acids

Chiral PNAs, particularly those based on D-lysine, have shown promising abilities in DNA recognition: it has been demonstrated that three adjacent D-lysine-based chiral monomers located in the middle of a PNA strand ("chiral box") can dramatically increase the specificity of recognition, both in terms of mismatch discrimination and of direction control, leading to an improved discrimination ability between an antiparallel and a parallel target.[12]

Therefore, chiral PNAs could be used whenever a very high specific recognition is required, such as in point-mutation recognition. However, at the moment two major obstacles are hampering the widespread use of chiral PNAs: the former involves the availability of chiral monomers with the different nucleobases, which have to be previously synthesized; the latter is the optical purity of the chiral residues

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in the final PNA strand, since the standard procedures used for the solid-phase synthesis of PNAs generally induce a partial racemization during the coupling steps.[13] The optical purity of D-Lys-containing chiral PNAs never exceeds 87−89% *ee* and, moreover, small variations in coupling reagents and conditions may strongly affect the percentage of racemization, making reproducibility quite a difficult task. The tendency to racemize can be ascribed to the fact that α-nitrogen atom of the amino acidic moiety in the chiral PNA monomers is involved in an amidic bond connecting the nucleobase (see Figure 1): it is well-known in peptide chemistry (for example in segment coupling condensation[14]) that this structural feature can lead to high percentages of racemization, due to the electron-withdrawing effect of the amidic group. As it has been demonstrated for phosphothioates[15] and recently by our group for other PNA-like molecules, ornithine nucleic acids (ONAs),[16] the DNA and RNA binding properties of chiral oligomers may be strongly affected by their optical purity. Moreover, the availability of optically pure PNAs may facilitate crystallization of chiral PNA-DNA duplexes,[17] thus allowing very useful insights into the recognition mode.

When approaching the solid-phase synthesis of peptide-like molecules, such as PNAs, the classical procedures require first the synthesis of the monomer suitably protected (usually by solution chemistry) followed by polymerization by solid-phase techniques. However, whenever the synthesis of the monomer is particularly complex, it is also possible to follow a different strategy, which can be called a "submonomeric" approach. A few examples of the submonomeric approach applied to the synthesis of PNA-like molecules, either on solid phase or in solution, have already been reported. The very first was reported by Richter et al.,[18] who synthesized a standard PNA by assembling very simple submonomeric units (bromoacetic acid, Moz-protected ethylenediamine and carboxymethylnucleobase) directly on the solid phase. Aldrian-Herrada et al.[19] reported the solid-phase synthesis of PNA monomers bearing a cisteamide residue, which were subsequently used in a standard solid-phase synthesis of a cysteamide-containing PNA. Di Giorgio et al.[20] followed different submonomeric approaches for the synthesis of standard achiral PNAs in solution. Van der Laan et al.[21] synthesized a poly-T-ONA in the solid phase, by the Boc strategy, where the actual submonomer used in the coupling step was a commercially available N^{δ}-Boc-N^{α}-Fmoc-ornithine. The coupling step was followed by Fmoc deprotection and coupling of carboxymethylthymine to the α-nitrogen atom, to yield the monomer required directly on the resin. After standard Boc deprotection, the cycle was repeated until completion of the oligomer. Quite interestingly, these authors reported a very high optical purity (> 99%) of a dimer obtained by this method, although measured by HPLC indirect determination of the diastereomeric excess. These data have been confirmed by us on a decamer synthesized according to the same procedure, the enantiomeric excess of the chiral residues being directly determined by chiral GC.[13] Very recently, a paper by Seitz and Kohler[22] reported a submonomeric solid-phase strategy for the rapid synthesis of fluorescence-labelled PNA oligomers. An N^{α}-Fmoc-protected aminoethylglycine backbone was inserted in a PNA strand, followed by the Fmoc deprotection and derivatization with a suitably modified carboxymethyladenine directly on a HYCRON resin.

However, at present, none of these procedures has substituted the standard Boc-strategy-based solid-phase syntheses of chiral PNAs. In this paper a suitable and robust submonomeric approach for the solid-phase Boc-based synthesis of chiral PNAs with high optical purity is described.

Results and Discussion

In order to rapidly synthesize different chiral PNAs with high optical purity, a submonomeric approach can be very useful, avoiding the cumbersome synthesis of chiral monomers and, at the same time, better preserving the optical purity at the α-carbon atom. By substituting an amidic group with a carbamate group the electron-withdrawing effect should be decreased, with a corresponding decrease in the acidity of the α-hydrogen atom. Therefore, we envisaged a submonomeric strategy in the context of a solid-phase Boc-based PNA synthesis, in which the chiral backbone, orthogonally N^{α}-Fmoc-protected (submonomer), was first linked to the growing chain on the resin, followed by Fmoc-deprotection and derivatization with the carboxymethylnucleobase.

Design and Synthesis of the Chiral D-Lys-PNA Submonomer

The submonomeric backbone was designed by using D-lysine as a chiral synthon, on account of the very interesting binding properties of the PNAs containing this amino acid.[12,23] In order to obtain a suitable submonomer to be used in solid-phase Boc-PNA synthesis, it was necessary to insert a suitable protecting group at the lysine N^{α}-position, orthogonal either to the Boc group or to the N^{ε}-lysine protecting group, i.e. 2-chlorobenzyloxycarbonyl (2-Cl-Z). The obvious choice was the 9-fluorenylmethoxycarbonyl (Fmoc) group, which can be cleaved by a mildly basic solution (20% piperidine in DMF). The target molecule is reported in Figure 2.

The first synthetic pathway was based on the synthesis of the *N′*-Boc-*O*-allyl chiral backbone **1**, starting from the commercially available N^{α}-Boc-N^{ε}-2-Cl-Z-D-Lys, by reaction with allyl bromide, Boc deprotection with TFA/DCM and reductive alkylation with Boc-aminoacetaldehyde, as already reported in the literature (Figure 3).[24]

The Fmoc group was first inserted in **1**, by reaction with Fmoc chloride, to obtain the N^{α}-Fmoc protected backbone **2**, then the allyl ester was removed by $[Pd(PPh_3)_4]$ in morpholine (Figure 4, reactions i and ii). The desired molecule **3** was obtained in good yield and with an extremely good enantiomeric excess (98.9%), as determined by chiral gas chromatography according to a direct method developed in our group.[25]

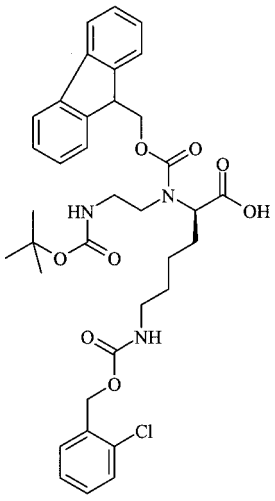

Figure 2. Target chiral submonomer based on D-Lys and fully protected (*N'*-Boc, N^{α}-Fmoc, N^{ε}-(2-Cl-Z)

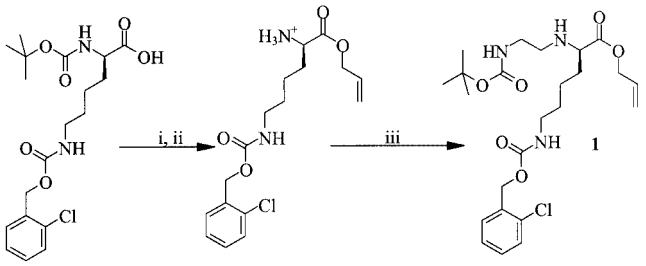

Figure 3. i) Allyl bromide, Cs_2CO_3, DMF, room temperature, 98% yield; ii) TFA, DCM, room temperature, 97% yield; iii) saturated $NaHCO_3$ solution, 91% yield; Boc-Gly-H, $NaBH_3CN$, CH_3COOH, CH_3OH, 0 °C, 60% yield; procedures performed as in ref.[23]

An alternative pathway was also investigated, based on the preliminary deprotection of the allyl group from **1**. The zwitterionic backbone **4** (Figure 4, reaction iii) was actually insoluble in different organic solvents (MeOH, CH_2Cl_2, THF, DMF), thus making the subsequent Fmoc protection to obtain **3** quite difficult. A good yield (88%) was obtained by dissolving the molecule in an aqueous solution of NaOH (1 M) and then adding the Fmoc-Cl (Figure 4, reaction iv). Unfortunately, under these conditions, the optical purity of the final target **3** was unsatisfactory (*ee* = 93.3%). Further experiments were done in order to determine the factors affecting racemization. The zwitterionic molecule **4** was dissolved in 1 M NaOH and allowed to stand for 1 h: quite surprisingly, the optical purity was very high (*ee* > 99%). In contrast, addition of Fmoc-Cl triggered the racemization process (*ee* = 95%) in the final product. These findings can be explained by assuming that the free carboxylate temporarily forms a mixed anhydride with the Fmoc group, thus increasing the inductive effect on the α-carbon atom and the relative acidity of the α-proton, leading to racemization, favored by the basic environment. The use of Fmoc-succinimidyl carbonate (Fmoc-OSu), which should lead to less mixed-anhydride formation, was unsatisfactory in terms of yield, given the lower reactivity of the derivative. The reaction was eventually improved, both in terms of the final yield and of the enantiomeric excess, by using a trimethylsilyl group as a temporary protection for the carboxylate. According to a similar reaction found in ref.[26], bis(trimethylsilyl)acetamide was added to a suspension of **4** in DCM. After the solid had dissolved, indicating the formation of a trimethylsilyl ester, Fmoc-Cl was added, together with the scavenging base triethylamine (Figure 4, reaction v). This reaction, quenched by MeOH, yielded the purified submonomer **3** (60%) with a very good optical purity (*ee* = 99.1%).

A different simplified strategy for the synthesis of **3** was also applied by synthesizing the methyl ester of the *N'*-Boc-protected backbone, rather than the allyl ester **1**. In fact, starting from the commercially available N^{α}-Boc-N^{ε}-2-Cl-Z-D-Lys, the cleavage of the Boc group and the synthesis of the methyl ester could be performed in a one-pot synthesis, simply by treating the molecule with a 1 M solution of $SOCl_2$ in MeOH. In this way the D-Lys(2-Cl-Z) methyl ester **5** was obtained. After standard reductive amination to obtain the methyl ester backbone **6** and hydrolysis by NaOH, the zwitterionic backbone **4** was obtained in high yield. The zwitterion backbone **4** was then Fmoc-protected via a trimethylsilyl ester, as reported above, to yield the target molecule **3**. The overall synthetic strategy is described in Figure 5. This strategy also turned out to be practically racemization-free (*ee* of the final submonomer = 99.1%).

A summary of the total yield and of the enantiomeric purity of the final target submonomer **3** for the three synthetic pathways proposed is reported in Table 1.

Synthesis of Chiral D-Lys-PNAs

With the chiral submonomer thus obtained, the solid-phase strategy was performed as reported in Figure 6.

Here, the crucial step is the derivatization of the N^{α}-(aminoethyl) group with the carboxymethyl base, which can be hampered by the steric hindrance of the secondary amine. In order to verify different synthetic conditions, several procedures for the synthesis of a dimeric PNA with one chiral and one achiral monomer, H-$T_{(D\text{-}Lys)}$T-NH_2 **7** (Figure 7), were investigated.

Starting from the resin preloaded with an achiral T monomer (Boc-T-MBHA-PS resin, 0.2 mmol/g), the Boc group was first removed by treatment with TFA, then the submonomer was coupled to different portions of the resin by using different coupling reagents, as reported in Table 2. In all cases the coupling was successful, as indicated by negative Kaiser tests.

All batches were treated with a TFMSA/TFA mixture, obtaining crude products which underwent GC analysis for the optical purity determination. The results are also reported in Table 2. The best enantiomeric excess, obtained either with the HBTU or HATU coupling reagent, was around 98% in both cases. It should be remarked that, when Lys-containing PNAs were synthesized by standard procedures and essayed by this method, the *ee* ranged from 79.0 to 87.6%.[27] Starting from the resin preloaded with the achiral T monomer and the chiral submonomer, the Fmoc group was removed by treatment with 20% piperidine in

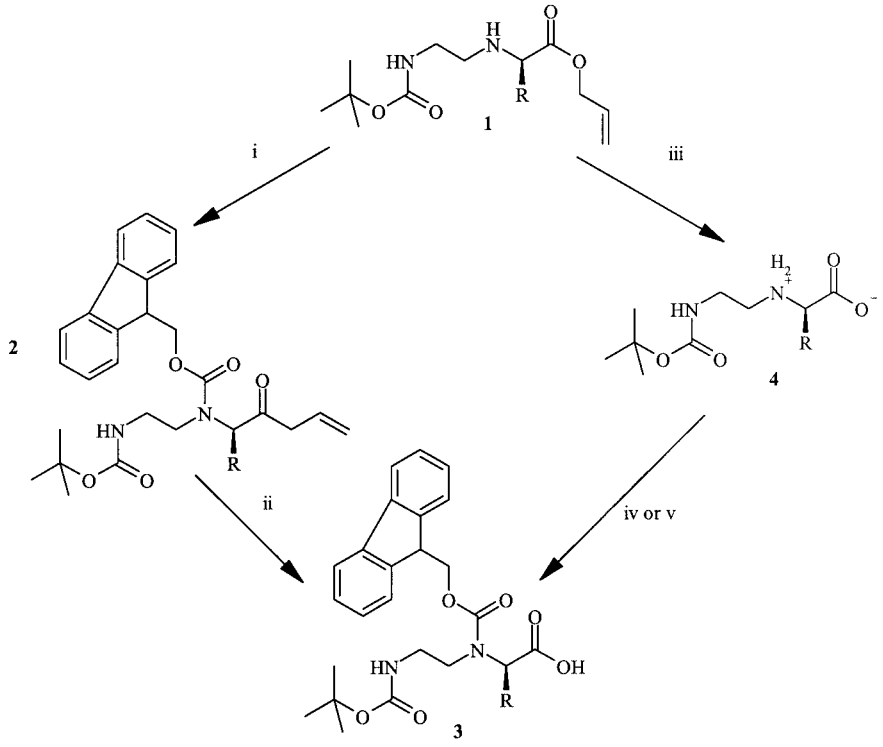

Figure 4. i) Fmoc-Cl, DIPEA, DCM, room temperature, 97% yield; ii) morpholine, $[Pd(PPh_3)_4]$, THF, room temperature, 80% yield; iii) morpholine, $[Pd(PPh_3)_4]$, THF, room temperature, 33% yield; iv) 1 M NaOH, Fmoc-Cl, THF, 88% yield; v) BTSA, DIPEA, Fmoc-Cl, DCM, room temperature, 60% yield; R = $-CH_2CH_2CH_2CH_2NH-$(2-Cl-Z)

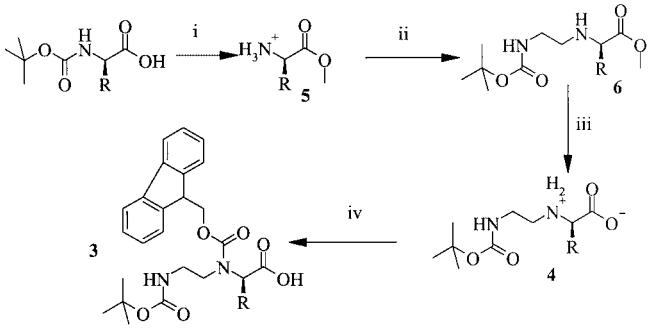

Figure 5. i) Thionyl chloride, CH_3OH, room temperature, quantitative yield; ii) saturated $NaHCO_3$ solution 88% yield; Boc-Gly-H, $NaBH_3CN$, CH_3COOH, CH_3OH, 0 °C, 64% yield; iii) 1 M NaOH, THF/H_2O, room temperature, quantitative yield; iv) BTSA, DIPEA, Fmoc-Cl, DCM, room temperature, 60% yield; R = $-CH_2CH_2CH_2CH_2NH-$(2-Cl-Z)

Table 1. Final yield (starting from commercially available N^{α}-Boc-N^{ε}-2-Cl-Z-D-Lys) and enantiomeric purity of the target submonomer **3** for the three synthetic pathways proposed

Strategy	Final yield	*ee*
Backbone allyl ester, *N*-protection then *C*-deprotection	40%	98.9%
Backbone allyl ester, *C*-deprotection then *N*-protection	10%	> 99%
Backbone methyl ester, *C*-deprotection then *N*-protection	34%	> 99%

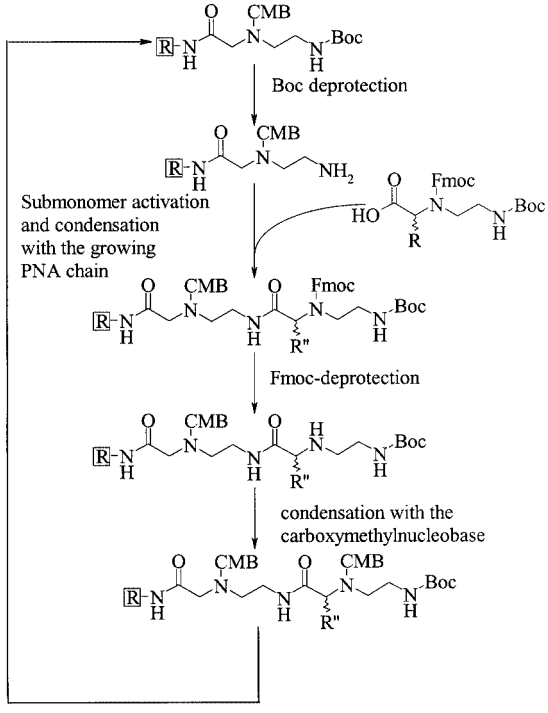

Figure 6. Submonomeric cycle for the insertion of a chiral monomer on a PNA chain on solid phase

DMF and the (carboxymethyl)thymine was coupled to the free secondary amino group. In this case, several attempts were also made on different portions of the resin mon-

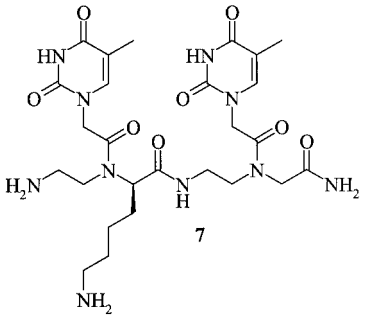

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Figure 7. Structure of the PNA dimer (**7**) H-$T_{(D\text{-}Lys)}$T-NH_2

Table 2. Coupling reagents used for the reaction of the chiral submonomer **3** with the resin and corresponding enantiomeric excess

Coupling reagent	Kaiser test after coupling	*ee*
HBTU /DIEA	negative	97.8%
HATU /DIEA	negative	97.6%
DIC/HOBt	negative	96.3%
TDBTU/DIEA	negative	94.5%

itoring the coupling reactions by a standard chloranil test. The results are reported in Table 3.

As shown, only the combination DCC/DHBtOH gave good results, as already observed during the synthesis of the monomers in solution.[23]

The portion of the resin which gave a negative chloranil test after coupling was subsequently treated with a TFMSA/TFA solution, yielding **7** as a crude product after Et_2O precipitation. HPLC-MS analysis of the crude product indicated a 93% yield of the desired compound, with only 3% of the backbone not coupled to the (carboxymethyl)thymine.

On account of these promising results, two longer chiral D-Lys-containing PNAs were synthesized by coupling the chiral D-Lys-submonomers with HBTU and, after Fmoc-deprotection, linking the carboxymethyl nucleobase with double coupling with DCC/DHBtOH. The achiral monomers in the sequences were coupled by standard procedures, either manually[28] or on an automatic peptide synthesizer.[29] The PNA sequences (**8**, **9**) are reported in Table 4, together with the optical purities (measured by GC analysis) obtained with the submonomeric strategy and with standard syntheses. Both PNAs were purified by HPLC and characterized by ESI-MS.

It is immediately evident that the enantiomeric excess is a little lower than that found in the dimeric PNA **7** (*ee* = 97.8%). This is probably due to the difficulty of coupling several adjacent bulky monomers: in fact, steric hindrance is likely to slow down the coupling reaction, thus making the epimerization more competitive. In any case, the optical purity is still very good when compared to the same PNAs synthesized by using the chiral full monomers and standard procedures. In fact, as shown in Table 4, PNA **8**, which has been used by our group in previous studies,[12] had been obtained by standard synthesis with an *ee* ranging from 52.0 to 89.0%, depending on the coupling reagent,[16] whereas PNA **9**, when synthesized by standard synthesis with HATU/collidine as coupling reagents, was obtained with only a disappointing 63.6% enantiomeric excess.

Therefore it is evident that the submonomeric approach proposed here constitutes an improvement for the chiral PNA synthesis, either in the efficiency, since one submonomer can be used for all the different nucleobases, and in the optical purity, since the enantiomeric excess are always reproducibly higher than those obtained by standard solid phase PNA synthesis.

Conclusions

In this paper we have described a submonomeric solid-phase strategy for obtaining chiral PNAs with high yield

Table 3. Coupling reagents and conditions used for the reaction of the backbone linked to the resin with (carboxymethyl)thymine (CMT)

CMT/backbone ratio	Coupling reagent	Preactivation	Coupling time	Chloranil test after coupling
1:1	HATU/DIEA	2 min	30 min	positive
2:1	HATU/DIEA	2 min	60 min	positive
7:1	DIC/DHBtOH	30 min	60 min	positive
7:1	DCC/DHBtOH	30 min	60 min	negative
7:1	TFFH/DIEA	10 min	30 min	positive

Table 4. Enantiomeric excess of the chiral PNAs synthesized by the submonomeric strategy compared with the enantiomeric excess of the same PNAs synthesized by standard syntheses with different coupling methods; chiral monomers are indicated between brackets in bold characters

PNA sequence	*ee* (%) standard	*ee* (%) submonomeric
H-GTAG(**$A_{D\text{-}Lys}$**)(**$T_{D\text{-}Lys}$**)(**$C_{D\text{-}Lys}$**)ACT-NH_2 (**8**)	89.0[a], 52.0[b]	94.4
H-TTCC(**$T_{D\text{-}Lys}$**)(**$C_{D\text{-}Lys}$**)(**$C_{D\text{-}Lys}$**)ACTG-NH_2 (**9**)	63.6[b]	94.3

[a] HBTU/diethylcyclohexylamine as coupling reagent. [b] HATU/collidine as coupling reagent.

and high enantiomeric excess. Different synthetic pathways towards a D-lysine-based submonomer to be used with the Boc strategy have been discussed, outlining optimal procedures. However, the route via a methyl ester is preferable since it combines fairly high yield with optimal optical purity.

Solid-phase syntheses of different chiral PNAs were performed by the submonomeric approach yielding chiral PNAs with high optical purity and high yield. The method appears to be excellent when only one chiral center is involved; in the syntheses of "chiral box" PNAs, where more adjacent bulky chiral centers are involved, this method allowed for the synthesis of chiral PNAs with the highest optical purity ever obtained.

The results presented here open the possibility to obtain highly optically pure chiral PNAs with a reasonable facility, improving the abilities of PNAs in the specific recognition of nucleic acids, and yielding more pure compounds for structural studies (X-ray crystallography, NMR spectroscopy) of the PNA-DNA complexes.

Experimental Section

General: Boc: *tert*-butoxycarbonyl; BTSA: bis(trimethylsilyl-acetamide); DCC: *N*,*N*′-dicyclohexylcarbodiimide; DCM: dichloromethane; DCU: *N*,*N*′-dicyclohexylurea; DHBtOH: 3-hydroxy-1,2,3-benzotriazin-4(3*H*)-one; DIPEA: diisopropylethylamine; DMF: *N*,*N*-dimethylformamide; DMSO: dimethyl sulfoxide; Fmoc: fluoren-9-yl-methoxycarbonyl; HATU: *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBTU: *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; MBHA-PS: methylbenzhydrylamine-polystyrene: Moz: 4-methoxybenzyloxycarbonyl; NMP: *N*-methylpyrrolidone; TFA: trifluoroacetic acid; TFFH: fluoro-*N*,*N*,*N*′-tetramethylformamidinium hexafluorophosphate; TFMSA: trifluoromethanesulfonic acid; THF: tetrahydrofuran; Z: benzyloxycarbonyl. All solvents and starting materials were used as commercially available. The PNA sequences are given from N to C.

Carboxymethyl Nucleobases: The (carboxymethyl)thymine and the Z-protected (carboxymethyl)adenine and (carboxymethyl)cytosine were synthesized as reported in ref.[30]

***N*′-Boc-Aminoethyl-*N*$^{\varepsilon}$-2-chloro-Z-D-lysine Allyl Ester (1):** The protected backbone was synthesized according to a literature procedure.[23]

***N*′-Boc-Aminoethyl-*N*$^{\varepsilon}$-2-chloro-Z-*N*$^{\alpha}$-Fmoc-D-lysine Allyl Ester (2):** *N*′-Boc-Aminoethyl-*N*$^{\varepsilon}$-2-chloro-Z-D-Lysine allyl ester (**1**) (0.56 g, 1.12 mmol) was dissolved in DCM together with DIPEA (0.29 mL, 1.67 mmol) and the solution was cooled to 0 °C. Fmoc-Cl (0.58 g, 2.24 mmol) was added and the mixture was stirred at room temperature for 2 h. The reaction was quenched with 1 M potassium hydrogen sulfate (60 mL). The organic layer was separated and the aqueous phase was washed with DCM (3 × 60 mL). The combined organic phases were dried with magnesium sulfate, filtered and the solvents evaporated under vacuum. The residue was purified by flash chromatography (ethyl acetate/hexane, 1:2). The product was a colourless oil. Yield: 0.73 g (97%). ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 1.2−1.6 (m, 6 H, $CH_2CH_2CH_2$ lysine side chain), 1.40 [s, 9 H, $(CH_3)_3$ Boc], 2.8−3.0 (m, 2 H, CH_2 aminoethyl group), 3.0−3.4 (m, 4 H, CH_2 aminoethyl group + CH_2 lysine side chain), 4.22 (s, 1 H, α-H), 4.4−4.6 (m, 5 H, CH_2CH Fmoc + OCH_2 allyl), 5.03 (s broad, 2 H, NH Boc + NH lysine side chain), 5.20 (s, 2 H, CH_2 2-Cl-Z-lysine), 5.1−5.3 (m, 2 H, CH_2 allyl), 5.8−5.9 (m, 1 H, CH allyl), 7.2−7.4 (m, 8 H, CH aromatic 2-Cl-Z-lysine + CH aromatic Fmoc), 7.62 (d, $^3J_{H,H}$ = 7.4, 2 H, CH aromatic Fmoc), 7.75 (d, $^3J_{H,H}$ = 7.3, 2 H, CH aromatic Fmoc) ppm. ^{13}C NMR (300 MHz, $CDCl_3$, 25 °C): δ = 23.4, 28.3, 28.6, 29.2, 39.5, 40.6, 46.5, 47.3, 60.4, 63.7, 65.8, 66.8, 79.2, 118.6, 119.8, 124.6, 126.7, 126.9, 127.6, 129.2, 129.4, 129.6, 131.6, 134.2, 134.3, 141.3, 143.8, 156.0, 156.1, 156.2, 170.9 ppm. MS-ESI (MeOH): calcd. for $C_{39}H_{47}ClN_3O_8$ [MH^+]: *m*/*z* = 720.3, found 720.0; calcd. for $C_{39}H_{46}ClNaN_3O_8$ [MNa^+]: *m*/*z* = 742.3, found 742.0. *ee* (chiral GC-MS according to the method reported in ref.[25]): 99.1%.

***N*′-Boc-Aminoethyl-*N*$^{\varepsilon}$-2-chloro-Z-*N*$^{\alpha}$-Fmoc-D-lysine (3) by Allyl Ester Deprotection:** *N*′-Boc-Aminoethyl-*N*$^{\varepsilon}$-2-chloro-Z-*N*$^{\alpha}$−Fmoc-D-Lysine allyl ester (**2**) (0.37 g, 0.51 mmol) was dissolved in THF (15 mL) at room temperature together with [Pd $(PPh_3)_4$] (0.06 g, 0.051 mmol). After stirring for 10 min, morpholine (0.44 mL, 5.1 mmol) was added to the mixture. After 5 min, the reaction was quenched with a 1 M potassium hydrogen sulfate solution (60 mL). The organic layer was separated and washed with 0.1 M potassium hydrogen sulfate (3 times). The combined organic phases were dried with magnesium sulfate, filtered and the solvents evaporated under vacuum. The residue was purified by flash chromatography (acetonitrile/methanol, gradient elution from CH_3CN 100% to CH_3CN/CH_3OH 50:50); the product was obtained as a white solid. Yield: 0.27 g (80%). *ee* (chiral GC-MS according to the method reported in ref.[25]): 98.9%.

***N*′-Boc-Aminoethyl-*N*$^{\varepsilon}$-2-chloro-Z-*N*$^{\alpha}$-Fmoc-D-lysine (3) by Zwitterion Protection:** Bis(trimethylsilyl)acetamide (BTSA) (0.3 mL, 1.2 mmol) and DIPEA (0.16 mL, 0.9 mmol) were added to *N*′-Boc-aminoethyl-*N*$^{\varepsilon}$-(2-chloro-Z)-D-Lysine (**4**) (0.27 g, 0.6 mmol) suspended in DCM (6 mL), with exclusion of water by a $CaCl_2$ drying tube. When the solution was nearly clear (10−15 min were usually required), Fmoc-Cl (0.31 g, 1.2 mmol) was added at 0 °C. After 10 min, the mixture was heated to room temperature and then stirred for 2 h. Methanol (2.4 mL) was carefully added and the mixture was stirred for an additional 15 min, diluted with DCM (20 mL), washed successively with a saturated $KHSO_4$ solution and brine, dried with magnesium sulfate and the solvents were evaporated. The residue was purified by flash chromatography (dichloromethane/methanol, 95:5). The product was obtained as a white foam. Yield: 0.29 g (60%). *ee* (chiral GC-MS according to the method reported in ref.[25]): 99.1%. Melting point 90−92 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 1.1−1.5 (m, 6 H, $CH_2CH_2CH_2$ lysine side chain), 1.32 [s, 9 H, $(CH_3)_3$ Boc], 2.9−3.1 (m, 6 H, CH_2 lysine side chain + CH_2CH_2 aminoethyl group), 4.1−4.4 (m, 4 H, α-H + CH_2CH Fmoc), 5.13 (s, 2 H, CH_2 2-Cl-Z-lysine), 7.1−7.3 (m, 8 H, CH aromatic 2-Cl-Z-lysine + CH aromatic Fmoc), 7.4−7.5 (m, 2 H, CH aromatic Fmoc), 7.67 (d, $^3J_{H,H}$ = 7.3, 2 H, CH aromatic Fmoc) ppm. ^{13}C NMR (300 MHz, $CDCl_3$, 25 °C): δ = 23.6, 28.3, 29.3, 29.7, 39.4, 40.7, 46.1, 47.2, 61.4, 63.6, 67.3, 79.4, 119.8, 124.8, 127.1, 127.6, 126.7, 129.1, 129.3, 129.6, 133.4, 134.5, 141.3, 143.9, 156.4, 156.5, 156.6 ppm. FT-IR (KBr): $\tilde{\nu}$ = 3422 (s), 2932 (m), 1700 (s), 1521 (w), 1251 (m) cm^{-1}. MS (ESI, CH_3OH): calcd. for $C_{36}H_{42}ClN_3NaO_8$ [MNa^+]: *m*/*z* = 702.3, found 702.0.

***N*′-Boc-Aminoethyl-*N*$^{\varepsilon}$-2-chloro-Z-D-lysine (4) by Allyl Ester Deprotection:** *N*′-Boc-Aminoethyl-*N*$^{\varepsilon}$-2-chloro-Z-D-Lysine allyl ester (**1**) (0.15 g, 0.30 mmol) was dissolved in THF (15 mL) at room temperature together with [Pd $(PPh_3)_4$] (0.035 g, 0.03 mmol). After stirring for 10 min, morpholine (0.38 mL, 4.3 mmol) was added to the mix-

ture. After 5 min, the reaction was quenched with a 1 M potassium hydrogen sulfate solution (60 mL). The organic layer was separated and washed with 0.05 M potassium hydrogen sulfate (3 times). The combined organic phases were dried with magnesium sulfate, filtered and the solvents evaporated under vacuum. The residue was dissolved in toluene and precipitated by adding hexane; the product was obtained as a white solid. Yield: 0.046 g (33%). *ee* (chiral GC-MS according to the method reported in ref.[25]): 98.5%.

N'-Boc-Aminoethyl-N^{ε}-2-chloro-Z-D-lysine (4) by Methyl Ester Deprotection: N'-Boc-Aminoethyl-N^{ε}-2-chloro-Z-D-lysine methyl ester (**6**) (0.22 g, 0.456 mmol) was dissolved in THF/H_2O, 1:1 (20 mL). NaOH (0.18 g, 4.56 mmol) was added and the mixture was stirred at room temperature for 15 h. After evaporation of the THF, the product was precipitated as a white solid at pH = 5.5, filtered and dried in vacuo. Yield: 0.21 g (quantitative). Melting point 229−230 °C. ^{1}H NMR (300 MHz, [D_6]DMSO, 25 °C): δ = 1.3−1.4 [m, 13 H, CH_2CH_2 lysine side chain + $(CH_3)_3$ Boc], 1.58 (s, 2 H, CH_2 lysine side chain), 2.6−2.7 (m, 2 H, CH_2 aminoethyl group), 2.98 (s, 2 H, CH_2 aminoethyl group), 3.0−3.1 (m, 3 H, α-H + CH_2 lysine side chain), 5.08 (s, 2 H, CH_2 2-Cl-Z-lysine), 7.3−7.4 (m, 4 H, CH aromatic 2-Cl-Z-lysine) ppm. FT-IR (KBr): $\tilde{\nu}$ = 3376 (s), 2937 (m), 1700(s), 1687 (s), 1540 (m) cm^{-1}. MS (ESI, CH_3OH): calcd. for $C_{21}H_{33}ClN_3O_6$ [MH^+]: *m*/*z* = 458.2, found 458.0; calcd. for $C_{21}H_{32}ClN_3NaO_6$ [MNa^+]: *m*/*z* = 480.2, found 480.0. *ee* (chiral GC-MS according to the method reported in ref.[25]): 99.1%.

N^{ε}-2-Chloro-Z-D-lysine Methyl Ester (5): N^{α}-Boc-N^{ε}-2-Chloro-Z-D-lysine-OH (1 g, 2.4 mmol) was dissolved in methanol (50 mL) with magnetic stirring. The mixture was cooled to 0 °C and thionyl chloride (3.65 mL, 50 mmol) was added dropwise. The solution was stirred for 24 h at room temperature. The methanol was evaporated to yield the product as a white solid. Yield: 0.9 g (quantitative). Melting point 108−110 °C. ^{1}H NMR (300 MHz [D_6]DMSO, 25 °C): δ = 1.3−1.4 (m, 4 H, CH_2CH_2 lysine side chain), 1.79 (dd, $^2J_{H,H}$ = 12.8, $^3J_{H,H}$ = 7.3 Hz, 2 H, CH_2 lysine side chain), 2.99 (dd, $^2J_{H,H}$ = 12.1, $^3J_{H,H}$ = 6.2 Hz, 2 H, CH_2 lysine side chain), 3.74 (s, 3 H, CH_3 methyl ester), 3.97 (t, $^3J_{H,H}$ = 6.3 Hz, 1 H, α-H), 5.08 (s, 2 H, CH_2 2-Cl-Z-lysine), 7.3−7.5 (m, 4 H, CH aromatic 2-Cl-Z), 8.49 (s broad, 3 H, NH lysine) ppm. ^{13}C NMR (300 MHz, CD_3OD, 25 °C): δ = 23.1, 30.3, 31.1, 41.2, 53.6, 53.9, 64.6, 128.1, 128.8, 129.3, 130.5, 134.2, 135.9, 158.7, 170.9) ppm. FT-IR (KBr): $\tilde{\nu}$ = 3408 (m), 2953 (m), 1748 (s), 1700 (m), 1254 (s) cm^{-1}. MS-ESI (CH_3OH): calcd. for $C_{15}H_{22}ClN_2O_4$ [MH^+]: *m*/*z* = 329.1, found 329.1.

N'-Boc-Aminoethyl-N^{ε}-2-chloro-Z-D-lysine Methyl Ester (6): N^{ε}-2-Chloro-Z-D-lysine-OMe (**5**) hydrochloric salt (0.5 g, 1.38 mmol) was dissolved in DCM (50 ml) and extracted with saturated sodium hydrogen carbonate (twice). The combined organic phases were dried with magnesium sulfate, filtered and the solvents evaporated under vacuum. Yield of the free amine: 0.4 g (88%). N^{ε}-2-chloro-Z-D-lysine-OMe (0.4 g, 1.2 mmol) was dissolved in methanol (10 mL) together with *N*-Boc-aminoacetaldehyde (0.19 g, 1.2 mmol). After stirring for 30 min at room temperature, the mixture was cooled to 0 °C and $NaBH_3CN$ (0.08 g, 1.2 mmol) and CH_3COOH (0.08 mL. 1.3 mmol) were added to the solution. After 1 h, the methanol was evaporated and the residue was dissolved in DCM (50 mL), washed with saturated potassium hydrogen sulfate (twice) and sodium hydrogen carbonate (twice). The combined organic phases were dried with magnesium sulfate, filtered and the solvents evaporated under vacuum. The crude product was purified by flash chromatography (eluent dichloromethane/methanol, 95:5) to give the pure compound as a colourless oil. Yield: 0.36 g (64%). ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 1.2−1.6 [m, 15 H, $CH_2CH_2CH_2$ lysine side chain + $(CH_3)_3$ Boc], 2.5−2.6 (m, 1 H, CH aminoethyl group), 2.7−2.8 (m, 1 H, CH aminoethyl group), 3.1−3.2 (m, 4 H, CH_2 aminoethyl group + CH_2 lysine side chain), 3.71 (s, 3 H, CH_3 methyl ester), 4.99 (s broad, 1 H, NH Boc), 5.21 (s, 2 H, CH_2 2-Cl-Z-lysine), 7.2−7.4 (m, 4 H, CH aromatic 2-Cl-Z-lysine) ppm. ^{13}C (300 MHz, $CDCl_3$, 25 °C): δ = 22.8, 28.3, 29.5, 32.8, 40.3, 40.7, 47.4, 51.7, 60.8, 63.7, 79.1, 126.8, 129.2, 129.4, 129.7, 133.4, 134.3, 156.1, 156.3, 175.6 ppm. FT-IR (liquid film): $\tilde{\nu}$ = 3443 (m), 3055 (s), 1715 (s), 1637 (s), 1422 (s), 1265(s), 739 (m) cm^{-1}. MS-ESI (CH_3OH): calcd. for $C_{22}H_{35}ClN_3O_6$ [MH^+]: *m*/*z* = 472.2, found 472.1. *ee* (chiral GC-MS according to the method reported in ref.[25]): > 99%.

PNA H-($T_{D\text{-}Lys}$)T-NH_2 (7): 70 mg of preloaded Boc-T-MBHA-PS resin (loading 0.2 mmol/g, obtained according to literature procedures[28]) was treated with TFA for Boc deprotection (5% *m*-cresol, 2 × 4 min). After washing with NMP and pyridine, the submonomer **3** (0.047 g, 0.07 mmol) was preactivated either with HBTU or HATU (0.027 g, 0.07 mmol) in NMP/pyridine for 2 min and coupled to the free amino group for 30 min. Successful coupling was monitored by a standard Kaiser test. After washing with NMP, the resin was treated with 20% piperidine in DMF for Fmoc deprotection (2 × 8 min). After deprotection, the resin gave a positive standard chloranil test. The (carboxymethyl)thymine (0.02 g, 0.1 mmol) was preactivated with DCC/DHBTOH (0.02 g, 0.1 mmol/0.017 g, 0.1 mmol) in NMP for 30 min, then the DCU was filtered off and the solution was poured onto the resin and left for 1 h. The procedure was repeated again. A negative chloranil test was taken as indication of successful coupling. Free PNA was cleaved from the resin by using a TFMSA/TFA (1:3) mixture (10% thioanisole + 10% *m*-cresol) and precipitated by Et_2O. Crude yield: 99%. ESI: calcd.: *m*/*z* = 621.3 [MH^+], 311.2 [MH_2^{2+}], found 620.9, 311.0. *ee* (chiral GC-MS according to the method reported in ref.[25]): 97.8%.

PNA H-GTAG$A_{D\text{-}Lys}T_{D\text{-}Lys}C_{D\text{-}Lys}$ACT-$NH_2$ (8): The synthesis was performed on 120 mg of a preloaded Boc-T-MBHA-PS resin (loading 0.2 mmol/g, obtained according to literature procedures[28]). Achiral monomers (Applied Biosystems) were inserted according to literature procedures.[28] Chiral monomers were inserted by submonomeric cycles according to the same procedures reported for **7**. Free PNA was cleaved from the resin by using a TFMSA/TFA (1:3) mixture (10% thioanisole + 10% *m*-cresol) and precipitated by Et_2O. Crude yield: 85%. HPLC purification was carried out on a semipreparative C_{18} column (250×10 mm Jupiter Phenomenex, 5 μm, 300 Å); eluent A: 100% water + 0.2% formic acid; eluent B: water/acetonitrile (60:40) + 0.2% formic acid; isocratic elution A/B (86:14), flow 4 mL/min. ESI: calcd.: *m*/*z* = 980.4 [MH_3^{3+}],735.6 [MH_4^{4+}], found 980.7, 735.5. *ee* (chiral GC-MS according to the method reported in ref.[25]): 94.4%.

PNA H-TTCC$T_{D\text{-}Lys}C_{D\text{-}Lys}C_{D\text{-}Lys}$ACTG-$NH_2$ (9): The synthesis was performed on 50 mg of a preloaded Boc-T-MBHA-PS resin (loading 0.2 mmol/g, obtained according to literature procedures[28]). Achiral monomers (Applied Biosystems) were inserted by using an ABI 433A peptide synthesizer (Applied Biosystems) with software modified to run the PNA synthetic steps. Chiral monomers were inserted by submonomeric cycles according to the same procedures reported for **7**. Free PNA was cleaved from the resin by using a TFMSA/TFA (1:3) mixture (10% thioanisole + 10% *m*-cresol) and precipitated by Et_2O. Crude yield: 87%. HPLC purification was carried out on a semipreparative C_{18} column (250×10 mm Jupiter Phenomenex, 5 μm, 300 Å); eluent A: 100% water + 0.2% formic acid; eluent B: water/acetonitrile (60:40) + 0.2% formic acid; gradient elution: from 100% A to 100% B in 25 min, flow

5 mL/min. ESI: calcd.: *m*/*z* = 780.1 [MH_4^{4+}], 624.3 [MH_5^{5+}], found 780.2, 624.2. *ee* (chiral GC-MS according to the method reported in ref.[25]): 94.3%.

Acknowledgments

The financial support from the EU (V Framework, "DNA-TRACK" project) and the Italian CNR (Progetto Finalizzato Biotecnologie and Agenzia 2001) is gratefully acknowledged.

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Received October 1, 2002
[O02542]