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Solid-Phase Synthesis of Cyclic PNA and PNA–DNA Chimeras

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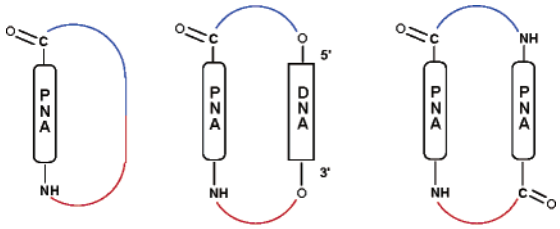
ABSTRACT



A new and versatile on-line automated solid-phase approach to obtain cyclic PNA (I and III) and cyclic PNA–DNA chimeras (II) in highly pure form has been developed. Starting from a Tentagel matrix functionalized with a 3-chloro-4-hydroxyphenylacetic linker, the synthesis of representative, new cyclic molecules by standard peptide and phosphoramidite-based chemistry has been achieved.

Cyclic oligonucleotides are highly specific ligands for DNA, RNA, and proteins offering a variety of biological applications.[1] Upon cyclization, oligonucleotides can adopt defined molecular structures in which the sugar−phosphate backbone provides a rigid framework to hold the nucleobases. The highly efficient molecular recognition properties of cyclic DNA are an incentive in probing the biological relevance of cyclic nucleic acid analogues as peptide nucleic acids (PNAs). In PNA, the sugar−phosphate backbone is replaced by an isosteric *N*-(2-aminoethyl)-glycine-based scaffold to which the nucleobases are linked through a carboxymethylene spacer.[2,3] These nucleic acid analogues offer several advantages, such as greater affinity and selectivity in the recognition of nucleic acids and proteins and better chemical and biological stability. PNAs show indeed some limitations due to their propensity to aggregation, low cellular uptake, ambiguous orientation, inability to activate the RNase H, and low solubility in aqueous media at relatively high concentrations. These shortcomings can be overcome by using PNA−DNA conjugates, often described as PNA−DNA chimeras.[4] The PNA−DNA chimeras, in which both types of monomeric units are present in the same chain, might combine the favorable hybridization behavior of PNA with the high water solubility of DNA.

Recently, short cyclic PNAs were synthesized as models to target natural biologically functional RNA loops.[5] However, the cellular uptake and pharmacodynamic properties of these molecules are still not optimal and further improvements in this regard are clearly desirable.

The number of papers reported in the literature on the synthesis of cyclic PNA oligomers is limited.[6−8] Only a few efforts have been made to address the solid-phase synthesis

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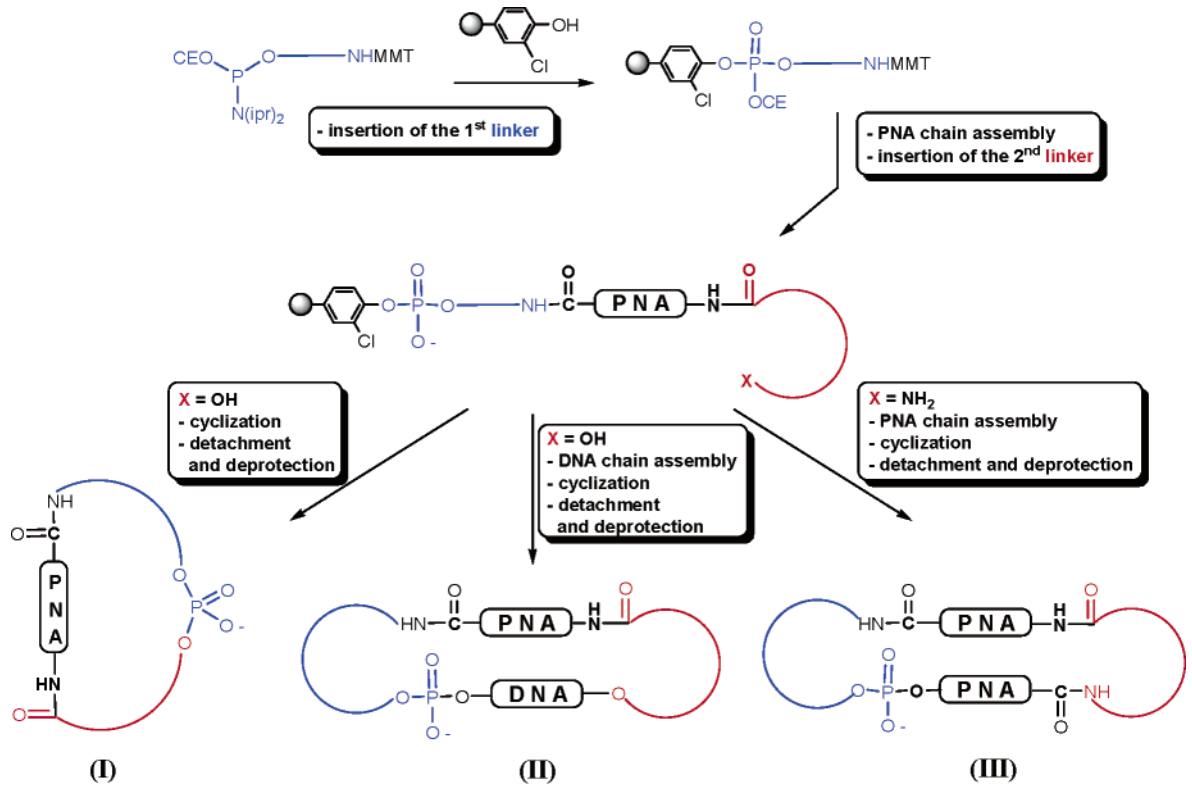
Scheme 1



of cyclic molecules containing a mixed PNA−peptide backbone,[9] and to the best of our knowledge, none have been made to address cyclic PNA−DNA chimeras. The synthetic approach already described for cyclic PNA is based on a fully protected backbone (FPB) strategy,[10] consisting of the elaboration of a fully protected linear poly(aminoethylglycinamide) chain using liquid-phase synthetic procedures. This requires as many different and orthogonal protecting groups as the number of different potential elongation sites and different base units introduced. In this approach, many steps and tedious purifications are required to isolate the desired molecules. In this frame, because of the inherent difficulty related to macrocyclizations and the subsequent purification steps, simply achieving the cyclic PNA molecule is per se a challenging task.

We report herein preliminary results on a versatile automated solid-phase strategy (Scheme 1) to obtain in a highly pure form cyclic PNA (**I** and **III**) and cyclic PNA−DNA chimeras (**II**), which represent a novel generation of cyclic model synthetic molecules. The convergent, on-line proposed strategy is based on the usage of a new solid support bearing two reactive functional groups: the transiently masked amine moiety (or hydroxy) on the chosen linker and the phosphodiester function. These can be exploited, respectively, for the PNA and DNA chain assemblies, exploiting standard peptide[11] and phosphoramidite-based[12] chemistry, and for the cyclization step.

Tentagel solid support, prederivatized with 3-chloro-4-hydroxyphenylacetic acid, leading to **1** (see Supporting Information) (Scheme 2), was functionalized with **2** (*link*$_1$) by classical phosphoramidite protocols, following a procedure recently developed for the solid-phase synthesis of 5′-phosphodiester and phosphoramidate monoester nucleoside analogues.[13] The conversion of the phosphite to phosphate triesters, affording support **3**, was achieved by a standard oxidizing treatment with I_2 in pyridine/H_2O/THF. The loading of resin **3**, determined by quantitation of the 4-methoxytriphenylmethyl (MMT) cation released from weighed amounts of the support upon acidic treatment, was always in the range 0.16−0.20 mequiv/g.

Support **3** contains two potentially reactive functional groups, i.e., the amino moiety on the aminohexane linker, transiently masked as an MMT amine, and the phosphotriester function, as a transiently masked form of a phosphodiester group. After MMT removal, with 1% dichloroacetic acid (DCA) in dichloromethane (DCM) solution, different PNA sequences were assembled by standard automated PNA−Fmoc synthesis[14] leading to supports **4a**−**d**. Fmoc tests monitoring the PNA assembly indicated, for each coupling cycle, yields not inferior to 97%. The piperidine

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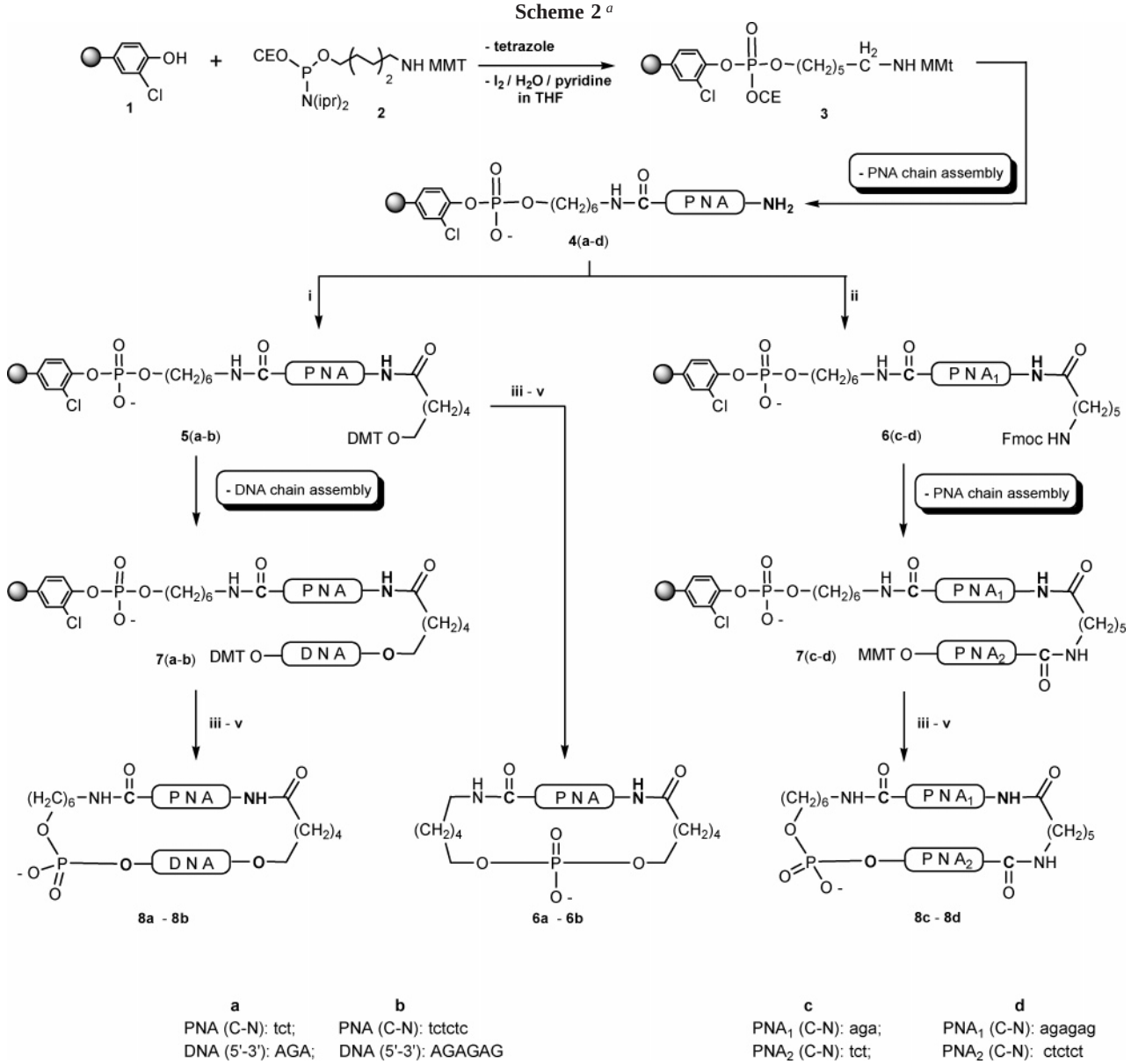
Scheme 2 [a]



[a] Reagents and conditions: (i) DCC, $DMTOCH_2(CH_2)_4COO^-Et_3NH^+$, *N*,*N*-diisopropylethylamine (DIPEA), pyridine, 6 h, rt; (ii) DCC, $FmocNHCH_2(CH_2)_4COOH$, DIPEA, pyridine, 6 h, rt; (iii) 1% DCA in DCM, 5 min, rt; (iv) 0.1 M MSNT in pyridine, 18 h, rt; (v) 0.1 M NaOH, dioxane/H_2O (1:1, v/v), overnight, 50 °C.

treatment, required in the PNA assembly protocol for Fmoc removal, led also to complete phosphate deprotection, as ascertained by ^{31}P NMR monitoring of the resin.

Supports **4a**−**d** have been exploited for the synthesis of cyclic PNA and PNA−DNA chimeras (**I**−**III**, Scheme 1). In the first set of experiments, supports **4a**,**b** (Scheme 2) were reacted in the presence of DCC with a flexible 6-hydroxyhexanoic acid (*link*$_2$), protected at the hydroxy function as 4,4′-dimethoxytriphenylmethyl ether (DMT ether), thus giving supports **5a**,**b**.

After detritylation with a 1% DCA solution in DCM, supports **5a**,**b** were reacted with 1-mesitylenesulfonyl-3-nitro-1,2,4-triazole (MSNT, 0.1 M in pyridine) at room temperature for 18 h, which provided the desired resin-bound cyclic derivatives. After alkaline treatment (0.1 M NaOH in H_2O/dioxane 1:1, v/v, 50 °C, overnight), the crude detached cyclic molecule was then purified by gel filtration chromatography on a Sephadex G-10 column eluted with H_2O/EtOH 1:1 (v/v). Reverse-phase (RP) HPLC analyses of the desalted samples showed in all cases a single peak, accounting for more than 85% of the total integrated area, corresponding to desired **6a** and **6b**, as confirmed by MALDI-TOF MS data (Table 1).

Such elevated purity is obtained because only the oligomers linked to the support through a phosphotriester function can be easily released, whereas the oligomers anchored through a phosphodiester bond are not cleaved from the resin upon alkaline treatment.[15]

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Table 1. Data for the Synthesized Cyclic Oligomers

entry	MS obsd/calcd for M	t_R (min)	OD[a]	overall yields[a] (%)
6a	1076.06 $[M - H]^-$ *1076.44*	8.7	31	14.5
6b	1847.23 $[M + H]^+$ *1844.75*	21.3	45	11.0
8a	2032.27 $[M - H]^-$ *2031.61*	10.3	60	10.7
8b	3772.89 $[M - H]^-$ *3771.08*	7.7	58	5.5
8c	1917.72 $[M + H]^+$ *1917.78*	8.4	38	6.8

[a] Referring to the isolated target compounds, as recovered after gel filtration, starting from 50 mg of support **3** with an average functionalization of 0.18 mequiv/g.

For the synthesis of cyclic PNA−DNA chimeras, starting from supports **5a**,**b**, the DNA tracts were assembled by standard phosphoramidite chemistry in the 3′−5′ direction, leading to **7a**,**b**. In all cases, DMT tests monitoring the ODN assembly indicated, for each coupling cycle, yields not inferior to 98%.

After detritylation (1% DCA in DCM), cyclization was carried out with MSNT (0.1 M) in pyridine at room temperature for 18 h. After cleavage and deprotection (0.1 M NaOH in H_2O/dioxane, 1:1, v/v, 50 °C, overnight), the crude detached material was then purified by gel filtration chromatography on a Sephadex G-10 column. Ion exchange HPLC analyses showed in all cases a single product, accounting for more than 90% of the total integrated area, corresponding to desired **8a** and **8b**, as ascertained by MALDI-TOF MS experiments (Table 1).

To prepare cyclic PNA-type **III** molecules, supports **4c**,**d** were reacted with FmocNH−$CH_2(CH_2)_4$COOH (*link*$_3$) in the presence of DCC in pyridine, thus giving supports **6c**,**d**. After Fmoc removal (20% piperidine in DMF), the second PNA tract was assembled by standard automated Fmoc synthesis,[14] leading to supports **7c**,**d**. In the last coupling step, we used a conveniently modified T PNA−OMMT monomer, previously described in the literature.[16] After detritylation (1% DCA in DCM), cyclization was achieved with MSNT (0.1 M) in pyridine at room temperature for 18 h. After cleavage and deprotection (0.1 M NaOH in H_2O/dioxane 1:1, v/v, 50 °C, overnight), the crude detached material was then redissolved in H_2O/EtOH (1:1, v/v) and purified by gel filtration chromatography on a Sephadex G-10 column. RP-HPLC analysis of desalted oligomer **8c** showed a single peak, identified by MALDI-TOF MS experiments as the desired cyclic compound. In an effort to synthesize larger cyclic PNA-type **III** molecules, the sequence **8d** (Scheme 2) was assembled following the same protocol used for **8c**. In no case could the desired molecule, still containing the same number of monomeric units as **8b**, be isolated. This result was interpreted as a consequence of a failure in the cyclization step, probably due to an unfavorable length-to-flexibility relationship, which in principle is the major intrinsic limit of the proposed synthetic strategy. Further optimization of the adopted linkers is currently in progress to allow the synthesis of cyclic PNA **III**-type molecules, carrying longer PNA stretches.

In a typical experiment, starting from 50 mg of support **3**, after oligomer assembly, cyclization, and detachment, 30−60 OD units of pure **6a**,**b** and **8a**−**c** could be recovered (see Table 1). As expected, cyclization yields dramatically depend on the size and nature of the linear oligomers.

The CD spectral properties of cyclic chimera **8b** were investigated in a phosphate buffer (100 mM NaCl, 10 mM K_2HPO_4, pH = 7.0, concentrated 1.5 μM) at 25 °C. The CD behavior of **8b** is similar to that typical of small cyclic oligonucleotides,[17] with a maximum at 250 nm and a minimum at 286 nm, confirming the cyclic nature of the chimera. Preliminary experiments to investigate the hybridization properties of the synthesized cyclic molecules were carried out for **6b** and **8b**. Duplex formation was monitored by UV thermal denaturation studies at 260 nm, mixing, in a 1:1 ratio, cyclic PNA **6b** or the cyclic chimera **8b** with the complementary DNA strand d($^{5'}$AGAGAG$^{3'}$) in a phosphate buffer at 3.5 μM. Melting and renaturation curves were always superimposable and showed, for the two investigated duplexes, typical S-shaped transitions with T_m = 49 °C and 52 °C, respectively. Similar experiments, carried out for comparison, mixing the DNA strand d($^{5'}$AGAGAG$^{3'}$) with the linear PNA (C)-tctctc-(N), gave for the resulting duplex T_m = 26 °C. These results confirmed that cyclic PNA-type **I 6b** and chimera **8b** recognize the complementary DNA sequence with largely enhanced affinity.

In conclusion, we reported here a simple on-line solid-phase method to obtain short-to-medium-sized cyclic PNA and cyclic PNA−DNA chimeras by exploiting standard peptide and phosphoramidite-based chemistry.

A set of representative new cyclic molecules have been prepared in a highly pure form, requiring a simple gel filtration chromatograpy for the final purification. Exploiting this synthetic scheme that allows us to employ linkers and sequences of a different size and nature, we synthesized a new class of cyclic PNA−DNA (**II**) chimeras. In particular, the cyclic PNA (**III**) and PNA−DNA (**II**) oligomers represent novel classes of potential synthetic dumbbells.

Further studies are currently in progress to extend this strategy to larger cyclic synthetic molecules, to optimize the linker's length, and to investigate their hybridization modes and structural biophysical properties.

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Supporting Information Available: General methods; functionalization of the Tentagel matrix; synthesis of support **3**; HPLC profile for **6b** and **8b**; MALDI spectra for **6a**, **6b**, and **8a**−**c**; UV melting curves for **6b** and **8b**, both mixed in a 1:1 ratio with the complementary DNA 6-mer d($^{5'}$AGAGAG$^{3'}$); CD spectrum of **8b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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