Synthesis of a Novel Pyrrolidine-based Peptide Nucleic Acid that Contains Tertiary Amines in the Main Chain and Its Internalization into Cells

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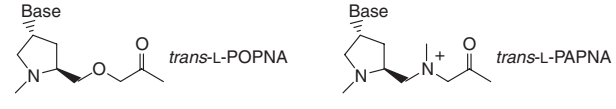
A novel thymine monomer of pyrrolidine-based peptide nucleic acid that contains tertiary amines in the main chain (pyrrolidine-based amino peptide nucleic acid = PAPNA) was synthesized. A mixed oligomer of two PAPNA(T) units and seven units of pyrrolidine-based oxypeptide nucleic acid (POPNA), was synthesized by a solid-phase method. The mixed oligomer was internalized into CHO cells more readily than the previous version of POPNA oligomers.

Peptide nucleic acids developed by Nielsen et al. (PNA) form more stable duplexes with the complementary nucleic acids than the corresponding hybrids between nucleic acids.[1] Thus, the PNAs are expected to work as effective antisense and antigene drugs. However, water solubility of PNA is not high enough for their practical applications. To improve water solubility, we developed an oxypeptide nucleic acid (OPNA) that contains ether linkages in the main chain.[2] The ether linkages also afforded high flexibility in the main chain and caused sharp melting curves with DNAs. However, OPNA did not show high affinity to RNAs. Recently, we reported a conformationally restricted version of OPNA that possesses a pyrrolidine ring in a monomer unit (pyrrolidine-based oxypeptide nucleic acids = POPNAs).[3] The two chiral centers on the pyrrolidine ring gave an opportunity to optimize the configuration for hybridization with DNAs and RNAs. Of the four stereoisomeric POPNAs, the *trans*-L-configurated POPNA (Scheme 1, left) formed the most stable duplex with RNA. Here, we report an attempt to provide the POPNA with an ability of internalization into cells by introducing tertiary amino groups into the main chain.

The structure of a tertiary amine-containing pyrrolidine-based peptide nucleic acid (PAPNA) is shown in Scheme 1 (right). At the physiological pH (7.4), the tertiary amino groups will be protonated and the cationic charges will facilitate the internalization into cells. Cell penetrating peptides (CPP)–PNA conjugates have been commonly used for the internalization.[4] However, the positively charged CPP will cause non-specific binding to nucleic acids. In this work, POPNAs containing the least number of cationic charges are designed to achieve cell internalization without scarifying their hybridization property.

The synthetic route of *trans*-L-PAPNA carrying a thymine base **11** is shown in Figure 1. First, a secondary amine of *trans*-L-hydroxyproline (**1**) was protected with a Boc group. The product **2** was reacted with ethyl bromide to give the corresponding ethyl ester **3**. Then, the configuration of the hydroxy group of the 4th position on the pyrrolidine ring was reversed by the reaction with formic acid with DEAD, followed by the treatment with aqueous ammonia (**4**). The hydroxy group of **4** was then protected with a *tert*-butyldiphenylsilyl (TBDPS) group (**5**). Reduction of *cis*-L-configurated compound **5** with $NaBH_4$ resulted in the corresponding alcohol **6**. Then, the latter was treated with CBr_4 to give the bromide **7**. Nucleophilic substitution of **7** with sarcosine *tert*-butyl ester gave the corresponding tertiary amine **8**. Removal of the TBDPS group with tetrabutylammonium fluoride (TBAF) gave a *cis*-L-configurated intermidiate **9**. The intermediate **9** was obtained in an overall yield 22% from **1**.



Scheme 1. Chemical structures of monomer units of *trans*-L-POPNA (left) and *trans*-L-PAPNA (right).

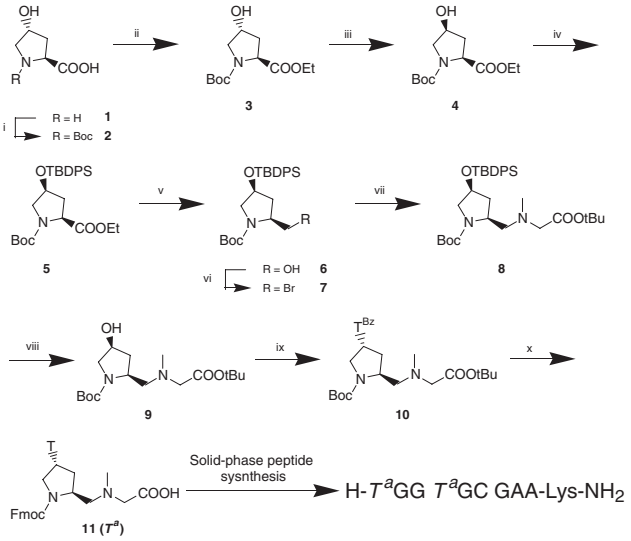


Figure 1. Synthetic route of Fmoc-protected thymine monomer of *trans*-L-PAPNA (T^a) and the sequence of PAPNA-containing oligomer. i) Boc_2O, $NaHCO_3$ in water:dioxane (1:1), overnight (95%); ii) a) DCHA in AcOEt, b) EtBr in DMF, overnight (94%); iii) a) formic acid, DEAD, Ph_3P in THF, overnight, b) 25% NH_3 aq in MeOH, 6 h (98%); iv) TBDPS–Cl, imidazole, DIEA in DMF, overnight (77%); v) $NaBH_4$ in EtOH, overnight (70%); vi) CBr_4, Ph_3P in THF, 3 h (85%); vii) H–Sar–O*t*Bu, Na_2CO_3 in DMF, 75 °C, 2 days (56%); viii) TBAF in THF, 4 h (99%); ix) N^3–Bz–Thymine, DEAD, Ph_3P in THF, overnight (crude); x) a) 30% HBr in AcOH, 30 min, b) Fmoc–OSu, $NaHCO_3$ in water:MeCN (1:1), overnight (60% at 2 steps).

The alcohol **9** was reacted with N^3-benzoylthymine under standard Mitsunobu conditions to give compound **10**. Then, compound **10** was treated with 30% HBr in acetic acid to remove the Boc, *tert*-butyl ester, and the N^3-benzoyl group. The amino group of free compound was protected again with an Fmoc group to give the final product **11** in an overall yield of 60% from the key intermediate. The new compound **11** was characterized

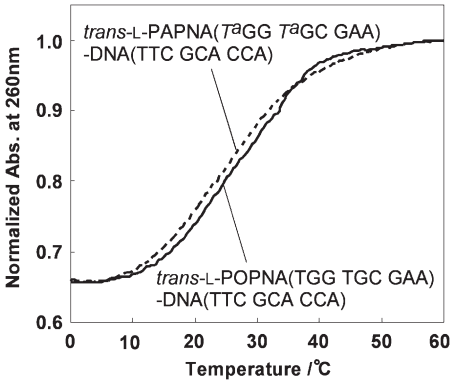


Figure 2. Temperature dependence of the absorption at 260 nm for equimolar mixtures of PAPNA 9-mer and the complementary DNA (dashed line), and that for POPNA 9-mer and the complementary DNA (solid line). [POPNA] = [PAPNA] = [DNA] = 5.0 μM in phosphate buffer (10 mM NaH_2PO_4, 0.1 M EDTA, 100 mM NaCl, pH 7.0).

by NMR (single species) and high resolution mass spectrometry (calcd $(M + H)^+ = 519.2165$, obsd. $(M + H)^+ = 519.2255$).

A mixed 9-mer POPNA oligomer containing two thymine PAPNA units (PAPNA 9-mer) was prepared by a solid-phase peptide synthesis (Fmoc/HATU). The base sequence was H–T^aGG T^aGC GAA–Lys–NH_2, where T^a indicates the PAPNA thymine monomer and G, C, and A indicates the corresponding POPNA monomers. As a noncharged control compound, a 9-mer POPNA oligomer without the PAPNA unit (POPNA 9-mer, H–TGG TGC GAA–Lys–NH_2) was also prepared. The crude oligomers were purified by preparative HPLC to single peaks and the purified oligomers were identified by MALDI-TOF mass spectroscopy (PAPNA 9-mer, calcd $(M + H)^+ = 2661.19$, obsd. $(M + H)^+ = 2659.39$; POPNA 9-mer, calcd $(M + H)^+ = 2635.13$, obsd. $(M + H)^+ = 2634.26$).

UV melting curve of an equimolar mixture of the PAPNA 9-mer (T^aGG T^aGC GAA) and the complementary DNA showed the melting temperature (T_m), 25.8 °C (Figure 2). The T_m was a little lower than that of the POPNA 9-mer (TGG TGC GAA) measured under the same conditions, 26.8 °C. Thus, the incorporation of PAPNA units into POPNA oligomer did not largely affect the stability of POPNA–DNA hybrid.

The internalization of the PAPNA oligomer into CHO cells was examined on a confocal laser-scanning microscopy.[5] The fluorescence image of the CHO cells after equilibration with the FAM-labeled PAPNA oligomer[6] is shown in Figure 3 (right). The oligomer was successfully taken up by the CHO cells. The intracellular location of the PAPNA was mostly confined in small vesicular compartments, but some fraction seems to be spread out from the vesicles. On the other hand, no internalization was observed for the FAM-labeled POPNA 9-mer under the same conditions (Figure 3, left). These results indicate that cationic PAPNA units in the oligomer facilitate the internalization into CHO cells. Internalization has been reported for peptide nucleic acids that carry cationic sequences like cell penetrating peptides.[4] However, since the long cationic peptides may cause nonspecific binding to DNAs and RNAs, an alternative method has to be searched for. This work indicates that the solubility-improved version of peptide nucleic acids can be internalized into cells by attaching the least number of cationic charges.

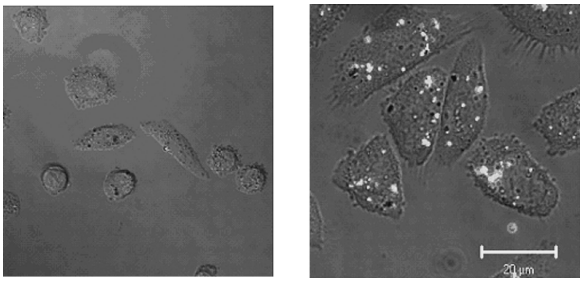


Figure 3. Confocal microscopy images of CHO cells cultured for 6 h in the presence of POPNA 9-mer (left) and PAPNA 9-mer (right). [PAPNA] = [POPNA] = 10 μM.

In summary, we successfully synthesized a novel peptide nucleic acid unit that carries a cationic charge, PAPNA. A 9-mer POPNA oligomer containing two PAPNA thymine units formed stable hybrid with DNA as well as a noncationic POPNA 9-mer. The cationic oligomer was effectively internalized into CHO cells.

References and Notes

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5 CHO cells were cultured in Dulbecco's modified Eagle's medium. The medium was supplemented with penicillin/streptomycin (10 μg/mL) and 10% fetal bovine serum. The cells were incubated at 37 °C under 5% CO_2 to give ca. 70% confluence. Then, subculture was performed on 35 mm glass-based dishes that were coated with poly(L-lysine). The cells were incubated at 37 °C under 5% CO_2 until 40–60% confluence. Before the cellular uptake, the cells were incubated at 37 °C for 6 h on a fresh medium containing FAM-labeled PAPNA 9-mer or FAM-labeled POPNA 9-mer. The final concentration of each peptide in the medium was 10 μM. The cells were then washed three times with PBS and examined on a confocal laser-scanning microscope without fixation.

6 The PAPNA oligomer was labeled with FAM–OSu after it was isolated from the resin. Thus, the FAM probe may be covalently attached on either the *N*-terminal of the Lys unit at the *C*-terminal (MALDI-TOF mass spectroscopy: calcd $(M + H)^+ = 3019.50$, obsd. $(M + H)^+ = 3019.58$).

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