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Synthesis of Peptide Nucleic Acids Containing Pyridazine Derivatives As Cytosine and Thymine Analogs, and Their Duplexes with Complementary Oligodeoxynucleotides

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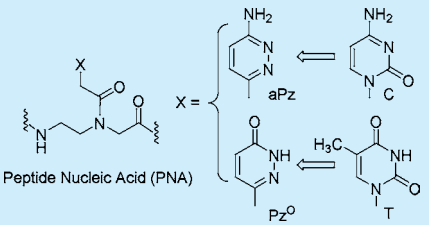
Supporting Information

ABSTRACT: Synthesis of peptide nucleic acids (PNAs) is reported with new pyridazine-type nucleobases: 3-aminopyridazine (aPz) and 1-aminophthalazine (aPh) as cytosine analogs, and pyridazin-3-one (Pz^O) and phthalazin-1-one (Ph^O) as thymine analogs. The PNAs having an aPz or a Pz^O formed duplexes with each complementary oligodeoxynucleotide forming a base pair with G or A, respectively, as evaluated by using UV melting analyses and circular dichroism (CD) spectra.



DNA and RNA contain pyrimidine nucleobases cytosine, thymine, and uracil, which form base pairs in various base-pairing motifs. Many pyrimidine base analogs have been reported for the development of nucleoside derivatives and oligonucleotide derivatives.[1−5] These analogs were designed from various six-membered heteroaromatic rings containing nitrogen atoms such as pyridine,[1] pyrimidine,[2] pyrazine,[3] and triazine[4] rings. The nucleotides and oligonucleotides incorporating these analogs exhibit unique physicochemical and biochemical properties. For example, a nucleoside analog incorporating 4-amino-2-oxo-1,3,5-triazine as a base exhibits antiviral and antirumor activities.[5] 2-Aminopyridine,[1f] pseudoisocytosine,[6] and 6-amino-pyrazin-2-one[3] base analogs incorporated into triplex-forming oligonucleotides (TFOs) have been used to recognize guanine residues of DNA duplex forming hydrogen bonds under neutral pH. 2-Aminopyrimidine[7] has also been used in TFOs to recognize cytosine residues within the DNA duplex. These studies suggest that pyrimidine base analogs are useful for the development of new artificial oligonucleotides and bioactive nucleoside or nucleotide analogs.

Here, we report the synthesis of new pyrimidine base analogs. Pyridazine-type nucleobases 3-aminopyridazine (aPz) and 1-aminophthalazine (aPh) as cytosine analogs, and pyridazin-3-one (Pz^O) and phthalazin-1-one (Ph^O) as thymine analogs (Figure 1), were synthesized and incorporated into peptide nucleic acids (PNAs) to study their base-pairing abilities. In previous studies, only three pyridazine derivatives have been described as pyrimidine analogs. For example, Furukawa et al. have reported the synthesis of a ribonucleoside derivative having a Pz^O residue as the base moiety.[8,9] However, its properties, including its base-pairing abilities, have not been reported. Additionally, Rozners et al.[10] and Nielsen et al.[11] reported PNAs harboring Pz^O as a thymine analog. These PNAs were designed to bind to the DNA duplex as TFOs, forming hydrogen bonds with the uracil of the U-A base pair. In these PNAs, the aminoethylglycine backbone and the Pz^O were connected via linkers derived from β-alanine[10] or 3-butenoic acid,[11] which are two atoms longer than the 1-oxyethylene linker used in the typical PNA unit shown in Figure 1. Therefore, the base-pairing properties of Pz^O in canonical PNAs have not yet been investigated. In contrast to these studies of Pz^O derivatives, the incorporation of aPz derivatives into any kind of artificial nucleic acid has not been reported. Here, we report the successful synthesis of PNAs containing pyridazine-type nucleobases and the evaluation of the base-pairing properties of these molecules.

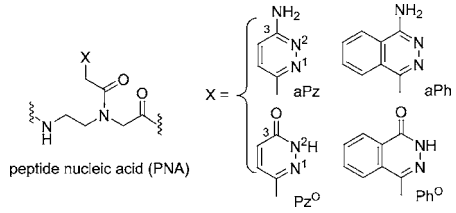


Figure 1. Structure of a PNA incorporating pyridazine-type nucleobases. The atoms of aPz and Pz^O are shown.

First, the electrostatic potential around 3-aminopyridazines was calculated and compared with that of cytosine. As shown in Figure 2, the shapes of the electrostatic potential surfaces around the amino groups and the endocyclic nitrogen atoms, N3 for cytosine (Figure 2, left) and N1 for aPz (Figure 2, second left), were similar. Although the exocyclic carbonyl oxygen of cytosine makes the surface around its C2═O2 position larger than that of the endocyclic N1 position of aPz, these surfaces were electrostatically negative. Romesberg and co-workers[12] studied the DNA polymerase reaction using DNA incorporating a pyridine-2-yl (2Py) and demonstrated that the Klenow fragment

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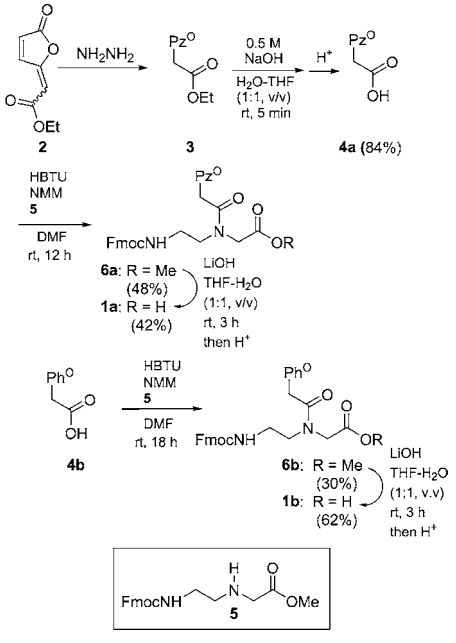
Figure 2. Electrostatic potential maps of cytosine, aPz, thymine, and Pz^O (from left to right) calculated at the HF/6-31G* level of theory. Chemical structures are overlaid to show approximate atom positions.

recognized the endocyclic nitrogen atom of 2Py instead of the 2-keto group of canonical pyrimidine nucleobases and catalyzed the strand extension from primer DNA having a 2Py at the 3′-terminal. Because the N1 of aPz and the nitrogen atom of 2Py were at the same endocyclic position, the α position to the glycosidic bond, it was expected that the N1 atom of aPz could also act as a mimic of the 2-keto group. We also calculated the electrostatic potential of thymine (Figure 2, second right) and Pz^O (Figure 2, right). Although the negative potential surface at around N1 of Pz^O was very small, the positions of the hydrogen donors and acceptors were similar.

The hydrogen bond energies between aPz and guanine were calculated. The base-pairing energies (ΔE) of aPz and guanine were −25 kcal/mol, which was less stable by 5 kcal/mol than the cytosine−guanine base pair. This was because of the lack of a hydrogen bond of the exocyclic carbonyl oxygen in the case of aPz guanine base pair. However, the ΔE of the aPz-guanine base pair was more stable than that of the thymine−adenine base pair by 7 kcal/mol. This result suggested that although there were only two apparent hydrogen bonds in aPz-guanine base pairs, there was an additional attractive dipole−dipole interaction between the amino group of the guanine and N1 of aPz. In addition, the hydrogen bond energy between Pz^O and adenine was calculated. The ΔE was −18.5 kcal/mol, which was slightly more stable than the thymine−adenine base pair (ΔE = −18.0 kcal/mol).

To incorporate these molecules into PNAs, we first synthesized a PNA unit having Pz^O (**1a**) (Scheme 1). Compound

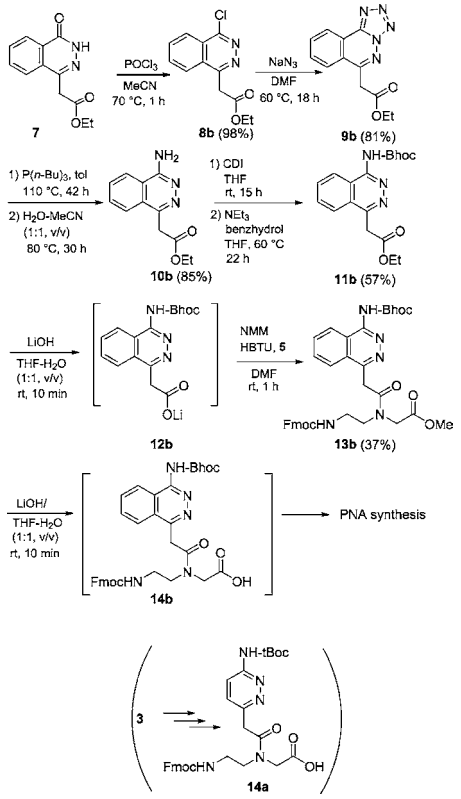
Scheme 1. Synthesis of a PNA Unit Incorporating Pz^O (1a) and Ph^O (1b)



2 was converted to **3** by the reaction with hydrazine.[13] The ethyl ester of **3** was hydrolyzed to the carboxylic acid **4a**. Compound **4a** was condensed with PNA backbone **5**[14] using hydroxybenzotriazolyltetramethyluronium hexafluorophosphate (HBTU) and *N*-methylmorpholine (NMM) as a condensing agent and a base, respectively, to give **6a**. Subsequently, compound **6a** was hydrolyzed to the PNA unit **1a**. Similarly, the PNA unit of Ph^O (**1b**) was synthesized using the carboxylic acid **4b**.[15]

The PNA unit having aPh (**14b**, Scheme 2) was also synthesized by using **7**.[15] Compound **7** was converted to the

Scheme 2. Synthesis of a PNA Unit Incorporating aPz (14a) and aPh (14b)



chloride **8b** using $POCl_3$. Subsequently, compound **8b** was treated with NaN_3, giving an azide compound that spontaneously cyclized to the tetrazole derivative **9b** as in the case of the reaction of 2-chloropyridine and sodium azide.[16] Then, the tetrazole moiety was reduced by treatment with P(*n*-Bu)$_3$, and successive hydrolysis gave the aPh derivative **10b**. After protection of the amino group with a benzhydryloxycarbonyl (Bhoc) group to give **11b**, the ethyl ester was hydrolyzed by using LiOH to give the lithium salt **12b** as an intermediate. Notably, if **12b** was converted to the corresponding free acid, it rapidly decomposed by decarboxylation, yielding benzhydryl (4-methylphthalazin-1-yl)carbamate. Therefore, lithium salt **12b** was directly condensed with **5** to give **13b** in 47% yield. Finally, compound **13b** was hydrolyzed to yield the PNA unit of aPh (**14b**). Because **14b** was not stable under purification on a silica gel column and storage, **14b** was immediately used for PNA synthesis without further purification after the removal of the residual **5** and other reagents by flash column chromatography. According to a similar procedure detailed in the Supporting Information (Scheme S1), the PNA unit of aPz (**14a**) was also synthesized starting from **3**. One of the differences between the

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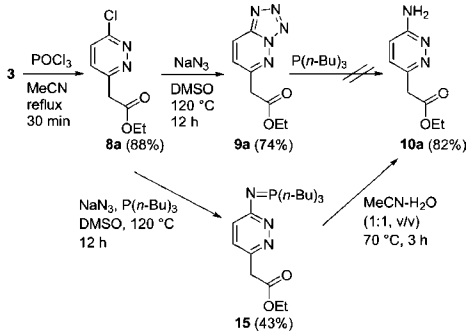
routes to **14a** and **14b** was that, in **14a**, the amino group was protected with a *tert*-butyloxycarbonyl (tBoc) group instead of a Bhoc group (Scheme 2 in the parentheses) because the Bhoc group introduced to the amino group of aPz was too labile under purification conditions. The other difference was that aPz derivative **10a** (Scheme 3) was directly synthesized from **8a** via

Scheme 3. Preparation of the Intermediate 10a



the intermediate **15** using a reaction in which sodium azide and $P(n\text{-Bu})_3$ coexisted.[17] Stepwise synthesis via **9a** failed because **9a** was too stable toward the reduction by $P(n\text{-Bu})_3$.

By using the PNA units **1a**, **1b**, **14a**, **14b** and commercially available PNA units and Fmoc-Lys-OH,[18] **PNA**s (Figure 3) were synthesized. After cleavage from the NovaSyn TGR resin,[19] the PNAs were purified using reversed-phase HPLC and characterized by ESI-TOF mass spectrometry.

^{H2N}AGTG[X]TCTAC-Lys^{CONH2}

PNA-aPz: X = aPz, **PNA-aPh**:X = aPh
PNA-C: X = C, **PNA-Pz^{O}**:X = Pz^{O}
PNA-Ph^{O}: X = Ph^{O}; **PNA-T**: X = T

$^{3'}$TCAC[Y]AGATG$^{5'}$

DNA-G: Y = G, **DNA-A**: Y = A, **DNA-C**: Y = C
DNA-T: Y = T

Figure 3. Sequences of PNAs and oligodeoxynucleotides used for hybridization studies.

Next, the hybridization of **PNA-aPz** and **PNA-aPh** with **DNA**s (Figure 3) were studied using thermal denaturation studies (Table S1).

The UV melting curve of **PNA-aPz** and **DNA-G** showed a transition at 40 °C, which was lower by 12 °C than that of the duplex of **PNA-C** and **DNA-G**, whose T_m was 52 °C. The lower T_m of **PNA-aPz** was because of one less hydrogen bond in comparison with the C−G pair. On the other hand, in the case of the duplexes with **DNA-A**, **DNA-C**, and **DNA-T**, the UV melting curves showed multiple transitions between 30 and 40 °C. PNA has been reported to form preorganized structures that melt at around 40 °C in the single-stranded state.[19] Therefore, the multiple transitions must be the overlap of the transitions of PNA-DNA duplexes and the single-stranded PNAs.

In the case of **PNA-aPh**, the T_m's of the duplexes were too low to be determined because the multiple transitions of the melting curves occurred at temperatures lower than 40 °C for any combination of **PNA-aPh** and the DNAs. The lower affinity of **PNA-aPh** and DNAs may be explained by the steric hindrance of the fused benzene ring of aPh, which interfered with the formation of stable duplexes.

Similarly, **PNA-Pz^{O}** formed a duplex with **DNA-A**, showing a single transition of the UV-melting curve at 36 °C, which was lower than that of the **PNA-T**/**DNA-A** duplex, for which the T_m was 45 °C. This result was unexpected because the molecular orbital calculation suggested that the Pz^{O}-A base pair was more stable than the T-A pair. One of the reasons might be that the stacking effects of the methyl group at position 5 and the keto group at position 2 of the thymine residue contributed to the higher stability of the duplex containing the T-A pair. For the mixtures of **PNA-Pz^{O}** with **DNA-G**, **DNA-T**, and **DNA-C** and the mixtures of **PNA-Ph^{O}** with **DNA-A**, **DNA-G**, **DNA-T**, and **DNA-C**, the transitions of the melting curves occurred below 30 °C, and the T_m's of the duplexes were not determined.

To study the structures of the complexes of PNAs and DNAs, we measured the circular dichroism (CD) spectra of **PNA-aPz** and **PNA-aPh** (Figure 4) and **PNA-Pz^{O}** and **PNA-Ph^{O}** (Figure

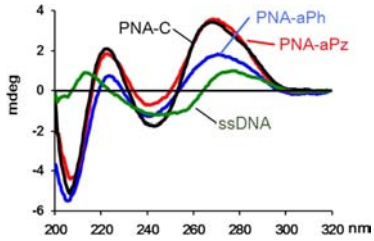


Figure 4. Circular dichroism (CD) spectra of **PNA-aPz** (red), **PNA-aPh** (blue), and **PNA-C** (black) with **DNA-G**. The green lines indicate the CD spectra of single stranded **DNA-G**. Conditions: 2.0 μM oligonucleotides, 10 mM sodium phosphate buffer (pH 7.0), 100 mM NaCl, 0.1 mM EDTA at 5 °C.

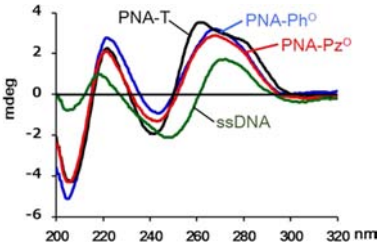


Figure 5. Circular dichroism (CD) spectra of **PNA-Pz^{O}** (red), **PNA-Ph^{O}** (blue), and **PNA-T** (black) with **DNA-A**. The green lines indicate the CD spectra of single-stranded **DNA-A**. Conditions: 2.0 μM oligonucleotides, 10 mM sodium phosphate buffer (pH 7.0), 100 mM NaCl, 0.1 mM EDTA at 5 °C.

5) at 5 °C in the presence of the complementary DNAs. As shown in Figure 4, **PNA-aPh**/**DNA-G** and **PNA-aPz**/**DNA-G** showed CD curves having positive bands at around 270 and 220 nm and a negative band at around 240 nm; these spectra were the same as those of the **PNA-C**/**DNA-G** duplex and different from those of single-stranded **DNA-G**. The single-stranded PNAs did not show any strong CDs. These peaks were characteristic of PNA-DNA duplexes[19,20] and suggested the formation of the PNA/DNA duplexes. In particular, the spectra of **PNA-aPz**/**DNA-G** and **PNA-C**/**DNA-G** were quite similar at around 270 and 220 nm. When the spectra of the **PNA-aPh**/**DNA-G** duplex and the **PNA-C**/**DNA-G** duplex were compared, we found that the peak intensities at 270, 240, and 220 nm were smaller in the case of **PNA-aPh**/**DNA-G** than in **PNA-C**/**DNA-G**. Because the shape of the CD spectrum is sensitive to the relative geometry of

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the nucleobases, these data suggested that there were small structural differences between **PNA-aPh/DNA-G** and **PNA-C/DNA-G**. Possibly, the base stacking around the aPh residue may change.

Similarly, the CD spectra of **PNA-Ph^O**, **PNA-Pz^O**, and **PNA-T** were compared in the presence of **DNA-A**. The mixture of **PNA-T** and **DNA-A** showed a major positive band at around 260 nm with a small shoulder at around 280 nm, a negative band at around 240 nm, and a positive band at around 220 nm. Similarly, **PNA-Ph^O/DNA-A** and **PNA-Pz^O/DNA-A** showed two positive bands at around 270 and 220 nm and a negative band at around 220 nm. These data suggested the formation of the PNA/DNA duplex even in the presence of modified bases such as Ph^O and Pz^O.

In conclusion, we successfully synthesized peptide nucleic acids incorporating aPz or aPh as a cytosine analog and Pz^O or Ph^O as a thymine analog. The PNA having one of these bases formed a duplex with complementary DNAs with lower affinity than that of the canonical PNA having thymine or cytosine. However, in the cases of **PNA-aPz** and **PNA-Pz^O**, these PNAs formed more stable duplexes with **DNA-G** and **DNA-A**, respectively, probably due to the formation of Watson−Crick-like base pairs. Thus, in terms of base recognition ability, our data suggested that aPz and Pz^O may replace cytosine and thymine, respectively. As reported by Romesberg and co-workers,[12] the endocyclic nitrogen at the α position relative to the glycosidic linkage can form a hydrogen bond with the amino acid residues of enzymes in DNA polymerase reactions. Therefore, nucleoside triphosphates having aPz or Pz^O as base surrogates may be good substrates for DNA polymerase reactions. If so, such molecules may be useful for the development of new anticancer or antiviral agents. We are now studying the synthesis of such nucleosides having aPz or Pz^O; the results will be reported elsewhere.

■ ASSOCIATED CONTENT

Supporting Information

The synthetic procedures for newly synthesized compounds and their NMR data are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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