Bioorg. Med. Chem. Lett. 39 (2021) 127850

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis and characterization of PNA oligomers containing preQ$_1$ as a positively charged guanine analogue

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ARTICLE INFO

Keywords:
Peptide nucleic acid
preQ$_1$
Strand invasion
Positive charge
Antigene

ABSTRACT

We report the synthesis of a peptide nucleic acid (PNA) monomer containing preQ$_1$, a positively charged guanine analogue. The new monomer was incorporated into PNA oligomers using standard Fmoc-chemistry-based solid-phase synthesis. The preQ$_1$ unit-containing PNA oligomers exhibited improved affinity for their complementary DNA through electrostatic attraction, and their sequence specificity was not compromised. It could be beneficial to incorporate preQ$_1$ into PNA oligomers instead of guanine when creating antisense/antigene agents or research tools.

Peptide nucleic acids (PNAs) are synthetic analogues of nucleic acids, which contain an *N*-(2-aminoethyl)glycine backbone instead of the sugar-phosphate backbone found in DNA.[1] PNA oligomers can specifically hybridize to complementary sequences through Watson-Crick base pairing. PNA oligomers bind tightly to DNA/RNA and exhibit high sequence specificity.[2–4] PNA oligomers are not substrates for nucleases or proteases[5] and do not bind to proteins non-selectively.[6] These unique properties make PNA oligomers attractive for researchers in the fields of chemistry, biology, pharmaceuticals, and medicine.

Since the first report about PNAs by Nielsen *et al.*, numerous modified PNAs have been synthesized.[7–9] In particular, there has been growing interest in positively charged PNAs, which have the potential to strengthen DNA/RNA binding, enhance cellular uptake, and reduce self-aggregation. Notably, it is expected that positive charges on PNA oligomers would enhance their ability to invade strands of duplex DNA, as it would stabilize PNA-DNA heteroduplexes and accelerate their hybridization with transiently single-stranded regions within duplex DNA.[10,11] The strand invasion ability of PNA oligomers makes them a very useful type of artificial DNA because strand invasion has a variety of applications, e.g., it can be used to cleave genomic DNA,[12] inhibit transcription,[13–15] and induce site-directed mutagenesis in vivo.[16]

We[17] and other groups[18–23] have reported chiral PNAs with cationic side chains in their backbones. In general, chiral PNAs with shorter cationic side chains exhibit higher binding affinity for DNA, which prompted us to study cationic PNAs containing simple nucleobases with short aminomethyl groups.

PreQ$_1$, 7-aminomethyl-7-deazaguanine, is a naturally occurring guanine-derived nucleobase found in the wobble position of tRNAs. It can be enzymatically converted to queuosine (Fig. 1).[24] There are three distinct classes of riboswitches that bind to preQ$_1$ and regulate the gene expression required for its biosynthesis or importation.[25] It has been shown that the incorporation of preQ$_1$ residues into DNA oligomers increased the thermal stability of DNA duplexes.[26] Since the primary amino group of preQ$_1$ is protonated under physiological conditions, the stabilizing effect was ascribed to the tethered NH_3^+ ion. Thus, the incorporation of preQ$_1$ was expected to cause a similar increase in affinity in PNA-DNA heteroduplexes. Here we report the synthesis of a preQ$_1$ PNA monomer and its incorporation into PNA oligomers. In addition, we describe the favorable hybridization properties of PNA oligomers containing preQ$_1$.

A tetra-Boc-protected *O*^{4}-*tert*-butyl ether derivative was designed as a protected preQ$_1$ monomer (Q monomer) for Fmoc-based solid-phase

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https://doi.org/10.1016/j.bmcl.2021.127850
Received 24 December 2020; Received in revised form 28 January 2021; Accepted 5 February 2021
Available online 2 March 2021

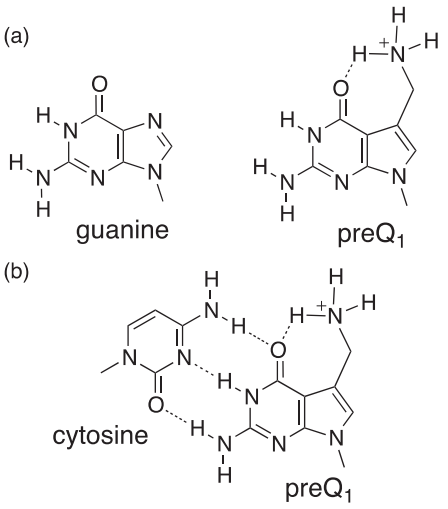


Fig. 1. (a) Chemical structures of guanine and preQ$_1$; (b) Plausible cytosine-preQ$_1$ base pair.

synthesis (Fig. 2). The synthesis of the Q monomer started from the preparation of phthalimido-protected preQ$_1$ (**4**), as shown in Scheme 1. The oxidation of *N*-(3-hydroxypropyl)phthalimide (**1**) with trichloroisocyanuric acid in the presence of catalytic amounts of (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) gave the aldehyde (**2**).[27] The α-bromination of **2** with trimethylsilyl bromide (TMSBr), and the subsequent condensation of the product with 2,6-diaminopyrimidine-4-one proceeded smoothly to afford the requisite intermediate **4** in a good yield (87%, 2 steps) and on a large scale.[28] The phthalimide group of **4** was removed by treating it with hydrazine to afford crude preQ$_1$ (**5**).

The direct conversion of **5** to the key intermediate **7** was not successful due to the poor solubility of **5** in organic solvents. To circumvent this problem, we employed a two-step protocol. First, the primary amino group of **5** was protected with a Boc group using Schotten-Baumann

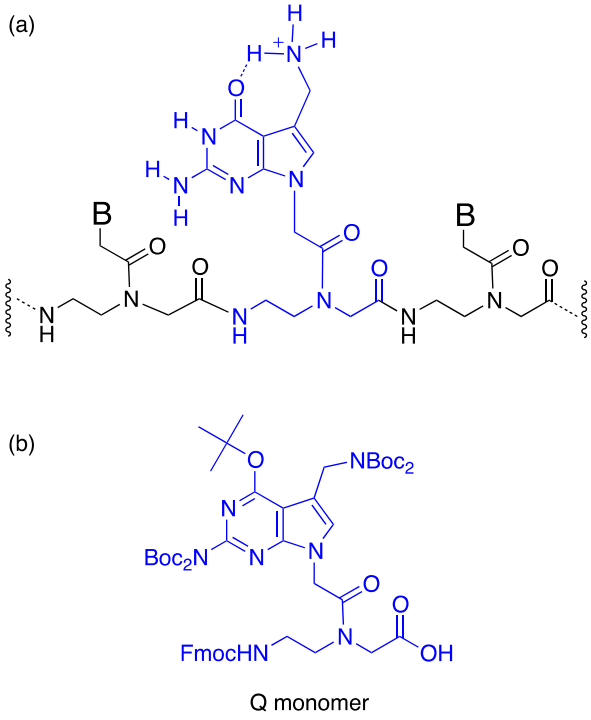
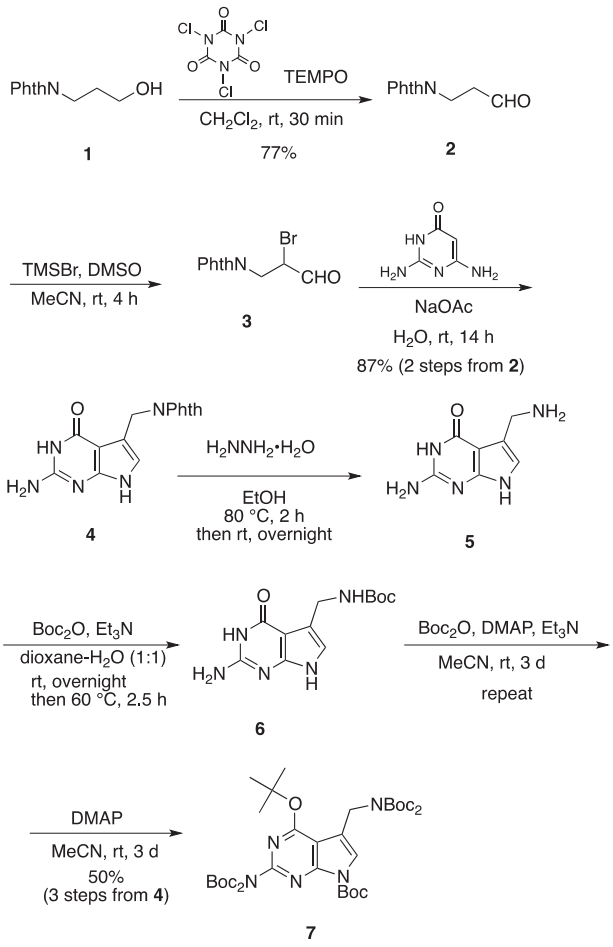


Fig. 2. (a) Structure of preQ$_1$ in a PNA oligomer; (b) Protected preQ$_1$ PNA monomer (Q monomer) used for Fmoc-based solid-phase synthesis (FmocSPPS).



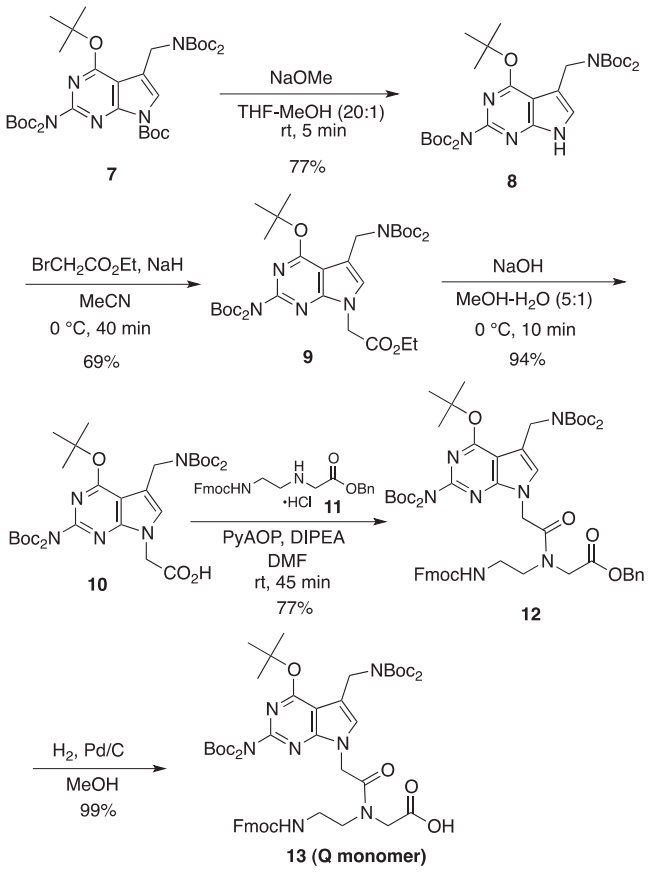
Scheme 1. Synthesis of the key intermediate **7**.

reaction conditions (Boc$_2$O and Et$_3$N in dioxane-H$_2$O) to improve the solubility of **5**. Second, the obtained mono-Boc-protected derivative **6** was converted to **7** as follows.

Crude **6** was suspended in MeCN and treated with excess Boc$_2$O and Et$_3$N in the presence of a catalytic amount of 4-dimethylaminopyridine (DMAP). After three days at room temperature, the suspension had changed to a clear solution. Since the reaction was incomplete at this stage, the fractions containing compound **7** and the intermediate compounds were collected by silica gel column chromatography. The residue was treated again with Boc$_2$O, Et$_3$N, and DMAP for three days. After being subjected to the same process as mentioned above and chromatographic separation, the residue was finally treated with DMAP alone to convert the remaining *N*-Boc intermediate to **7**.[29] The multi-Boc-protected derivative **7** was obtained in a 50% yield from **4** (3 steps).

The conversion of **7** to the protected preQ$_1$ monomer is shown in Scheme 2. The Boc group at the pyrrole NH was selectively removed by sodium methoxide to give tetra-Boc preQ$_1$ (**8**) (yield: 77%). The pyrrole NH of **8** was alkylated with ethyl bromoacetate to afford compound **9** (yield: 69%). Hydrolysis of the ethyl ester group of **9** with aqueous NaOH in MeOH-H$_2$O provided **10** in a 94% yield. Then, **10** was coupled with the backbone **11**[30] using (7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP) in DMF to afford the monomer benzyl ester **12** in a 77% yield. The Fmoc-protected Q monomer **13** was obtained in a 99% yield by catalytic hydrogenation of **12**.

Using standard Fmoc-chemistry-based solid-phase synthesis, one or three Q monomers were incorporated into PNA oligomers (Table 1; **P2**, **P3**). A 10-mer mixed-sequence PNA oligomer (**P1**), H-GTAGAGCACT-*Lys*-NH$_2$, was used as a reference. The oligomers **P1**–**P3** were manually



Scheme 2. Completion of the synthesis of the Q monomer.

Table 1
PNA oligomer sequences and their molecular weights.[a]

PNA	Sequence[b]	Molecular formula	Calcd. for [M + 4H]$^{4+}$	Found
P1	H-GTAGAGCACT-^{L}Lys-NH_2	$C_{114}H_{147}N_{63}O_{30}$	720.5551	720.5522
P2	H-GTAGA**Q**CACT-^{L}Lys-NH_2	$C_{116}H_{151}N_{63}O_{30}$	727.5629	727.5600
P3	H-**Q**TA**Q**A**Q**CACT-^{L}Lys-NH_2	$C_{120}H_{159}N_{63}O_{30}$	741.5786	741.5768

[a] Obtained using ESI orbitrap mass spectrometry; [b]Written from N to C terminal. PNA oligomers terminate in a lysine amide; **Q** = preQ$_1$ unit.

synthesized using 5 molar equiv. of the monomer, 4.5 molar equiv. of 1-(bis(dimethylamino)methylene)-1*H*-[1,2,3]triazolo[4,5-*b*]pyridine-1-ium 3-oxide hexafluorophosphate (HATU) as a coupling agent, and *N*-methyl-2-pyrrolidone (NMP) as a solvent. In addition to the Q monomer, commercial Fmoc/benzhydryloxycarbonyl (Bhoc)-protected monomers were also used. The Q monomer was very soluble in NMP and its incorporation into the PNA oligomers proceeded smoothly. After the completion of the oligomer synthesis, the resin was treated with trifluoroacetic acid-*m*-cresol-H_2O (83.3:13.9:2.8) to deprotect and cleave the oligomer from the resin. Then, the oligomers were subjected to high-performance liquid chromatography-based purification. The purified PNA oligomers were characterized by electrospray ionization orbitrap mass spectrometry. Table 1 shows the obtained PNA oligomers.

To investigate the effects of the introduction of the tethered NH_3^+ ion on the thermal stability of PNA-DNA heteroduplexes, the melting temperatures (T_m) of the duplexes formed between the PNA oligomers and complementary DNA were measured in UV-melting experiments. The melting profiles of the PNA-DNA heteroduplexes showed sigmoid curves (Fig. 3). The associated T_m values are listed in Table 2. As expected, the cationic amino group of preQ$_1$ improved the thermal stability of PNA-DNA heteroduplexes. The incorporation of a single Q monomer into PNA (**P2**) resulted in an increase in the T_m of the PNA-DNA duplex of 5.1 °C. The incorporation of three units of preQ$_1$ into PNA (**P3**) induced a further increase in the T_m of the PNA-DNA duplex; i.e., an increase of 18.3 °C (6.1 °C per modification), relative to the T_m of the duplex involving the reference PNA (**P1**). The stabilizing effect of the preQ$_1$ units was smaller at higher salt concentrations. All T_m values decreased as the NaCl concentration increased because the salt inhibited the electrostatic attraction between the phosphates and protonated amino groups. However, preQ$_1$ significantly stabilized the PNA-DNA duplexes, even at higher salt concentrations. It is likely that the preQ$_1$ modification of PNA oligomers enhances the hybridization stability of PNA-DNA duplexes to a greater extent than the effect of the preQ$_1$ modification of DNA on the hybridization stability of DNA duplexes.[26]

Using the PNA oligomers **P1** and **P3**, we also evaluated the base discrimination ability of preQ$_1$ by comparing the T_m values of matched and mismatched PNA-DNA duplexes. The mismatched duplexes contained a single-base mismatch opposite the guanine or preQ$_1$ site (Table 3). Although electrostatic attraction is generally considered to cause non-specific binding, **P3** exhibited excellent sequence selectivity even at low salt concentrations. The mismatch discrimination displayed by the preQ$_1$-containing PNA oligomers was somewhat better than that exhibited by the natural guanine-containing PNA oligomer. The incorporation of preQ$_1$ improved the DNA binding affinity of the modified PNA oligomers without compromising their sequence specificity.

In summary, we synthesized an Fmoc-protected preQ$_1$ PNA monomer (Q monomer) and incorporated it into PNA oligomers. The newly synthesized monomer exhibited excellent solubility in organic solvents and was successfully used for standard Fmoc-based solid-phase synthesis. The incorporation of a single preQ$_1$ unit into a PNA oligomer resulted in the oligomer's affinity for DNA being enhanced (its T_m increased by 5 °C) at a low salt concentration. The incorporation of three units of preQ$_1$ further enhanced the affinity of the oligomer for DNA (its T_m increased by 18 °C). Even at higher salt concentrations, preQ$_1$ stabilized PNA-DNA duplexes. We now possess a positively charged guanine analogue, and attempts to synthesize a cationic cytosine analogue are underway at our laboratory. Recently, a report on PNA containing N^7-methylguanine[31] has further prompted us to expand the scope of preQ$_1$-containing PNA oligomers. Experiments to evaluate the effects of preQ$_1$ on the cellular uptake of PNA oligomers and on the strand invasion of duplex DNA by PNA oligomers are currently ongoing at our laboratory.

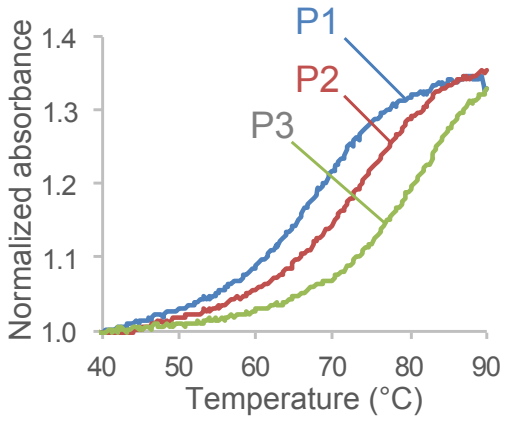


Fig. 3. UV-melting curves of the PNA-DNA hybrid duplexes at a strand concentration of 5 μM each. Samples were prepared in 10 mM sodium phosphate buffer containing 1 mM EDTA (pH 7.4).

Table 2
T_m (°C) values of the hybrid duplexes of PNA-DNA.[a]

	[NaCl]		
	0 mM	150 mM	1000 mM
P1	69.3	61.8	60.6
P2	74.4	63.6	57.5
P3	87.6	67.1	62.5

DNA = 3′-CATCTCGTGA-5′.

[a] The concentration of each strand was 5 μM. The strands were prepared in 10 mM sodium phosphate buffer containing 1 mM EDTA (pH 7.4) and the indicated concentrations of NaCl.

Table 3
T_m (°C) values of mismatched duplexes of PNA-DNA.[a]

	Fully matched DNA[b]	Mismatched DNA[c]	ΔT_m[d] (°C)
P1	69.3	40.4	−28.9
P3	87.6	56.5	−31.1

[a] The concentration of each strand was 5 μM. The strands were prepared in 10 mM sodium phosphate buffer containing 1 mM EDTA (pH 7.4).

[b] Fully matched DNA = 3′-CATCTCGTGA-5′.

[c] Mismatched DNA = 3′-CATCT<u>A</u>GTGA-5′ (with a G:A or Q:A base pair mismatch).

[d] ΔT_m indicates the difference between the T_m values of the fully matched duplex and the mismatched duplex.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was supported by Grants-in-Aid for Scientific Research Grant Numbers 18K06557 to T.S. and 18K06556 to A.K. from the Japan Society for the Promotion of Science. We thank Dr. Yusuke Akagi (Teikyo Heisei University) for his assistance with the NMR data analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2021.127850.

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