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PNA monomers fully compatible with standard Fmoc-based solid-phase synthesis of pseudocomplementary PNA

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ABSTRACT

Here we report the synthesis of new PNA monomers for pseudocomplementary PNA (pcPNA) that are fully compatible with standard Fmoc chemistry. The thiocarbonyl group of the 2-thiouracil (sU) monomer was protected with the 4-methoxy-2-methybenzyl group (MMPM), while the exocyclic amino groups of diaminopurine (D) were protected with Boc groups. The newly synthesized monomers were incorporated into a 10-mer PNA oligomer using standard Fmoc chemistry for solid-phase synthesis. Oligomerization proceeded smoothly and the HPLC and MALDI-TOF MS analyses indicated that there was no remaining MMPM on the sU nucleobase. The new PNA monomers reported here would facilitate a wide range of applications, such as antigene PNAs and DNA nanotechnologies.

Peptide nucleic acid (PNA) is a synthetic analogue of DNA in which the sugar-phosphate backbone is replaced by an *N*-(2-aminoethyl)glycine backbone.[1] PNAs hybridize to complementary sequences by Watson-Crick base pairing with high affinity and stringent sequence selectivity.[2] PNAs are resistant to nucleases and proteases[3] and have a low affinity for proteins.[4] These properties make PNAs an attractive agent for biological and medical applications.

The most notable feature of PNA is its strand invasion ability. The first example reported was triplex invasion by homopyrimidine PNAs that target homopurine tracts within duplex DNA.[1] In 1999, Nielsen et al. described pseudocomplementary PNAs (pcPNAs) that resolve limitations on sequence choice. pcPNAs are defined as pairs of complementary PNA oligomers that do not interact with each other.[5] The reported pcPNAs possessed 2,6-diaminopurine (D) and 2-thiouracil (sU) instead of natural adenine and thymine, respectively. Steric hindrance between the 2-amino group of D and the 2-thiocarbonyl group of sU destabilizes PNA-PNA duplexes (Fig. 1a). Although pcPNAs cannot form a stable PNA-PNA duplex, they have the ability to hybridize to complementary DNA sequences. Thus, pcPNAs target both strands of duplex DNA simultaneously and achieve sequence-specific recognition of duplex DNA using a double-duplex invasion mechanism (Fig. 1b). Since pcPNAs can bind to any sequence having at least 40% A + T content, more than 83% of all 10-mer sequences may be targeted.

Applications for double-duplex invasion by pcPNAs include the inhibition of RNA polymerase,[5] methylase,[6] and restriction enzymes.[6,7] Furthermore, pcPNAs have been used to make double-stranded DNAs vulnerable to metal-catalyzed hydrolysis,[8] to induce gene correction,[9] and as an actuator to trigger structural changes in the architecture of DNA in dynamic DNA nanotechnology.[10] Despite these promising characteristics of pcPNAs, they have not been as widely used as expected. This is mainly because pcPNAs are not easily available. The Boc/Cbz and Fmoc/Bhoc PNA monomers are both commercially available and can be used in the synthesis of conventional PNAs through Boc chemistry and Fmoc chemistry, respectively. However, monomers for sU and D are not commercially available. In addition, the solid-phase synthesis of pcPNA is only achieved by Boc chemistry due to the lack of the Fmoc monomers of sU and D.[5,11] In Boc-based PNA

Abbreviations: Bhoc, benzhydryloxycarbonyl; HBTU, 1-[bis(dimethylamino) methylene]-1*H*-benzotriazolium hexafluorophosphate 3-oxide; PyAOP, 7-azabenzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)-phosphonium hexafluorophosphate.

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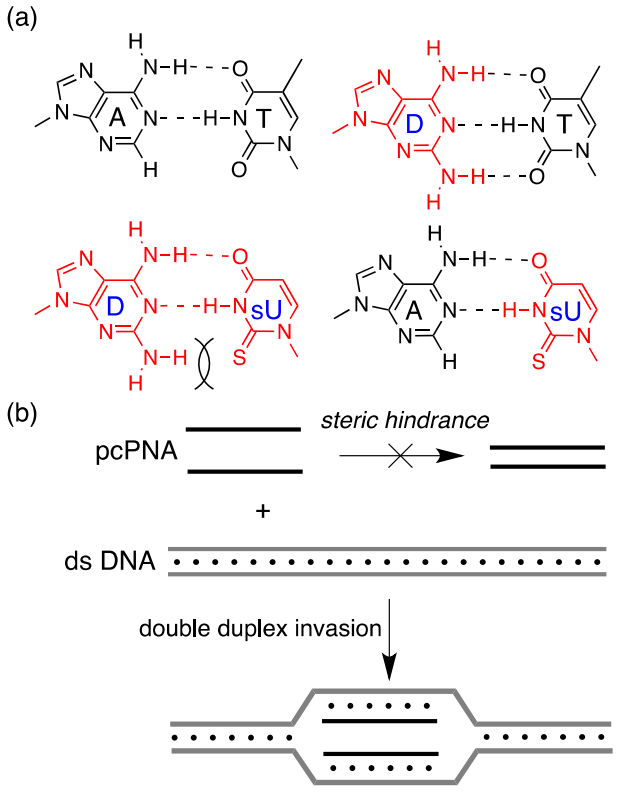


Fig. 1. (a) Chemical structures of A-T, D-T, D-sU, and A-sU paired nucleobases. (b) Double-duplex invasion by pcPNA.

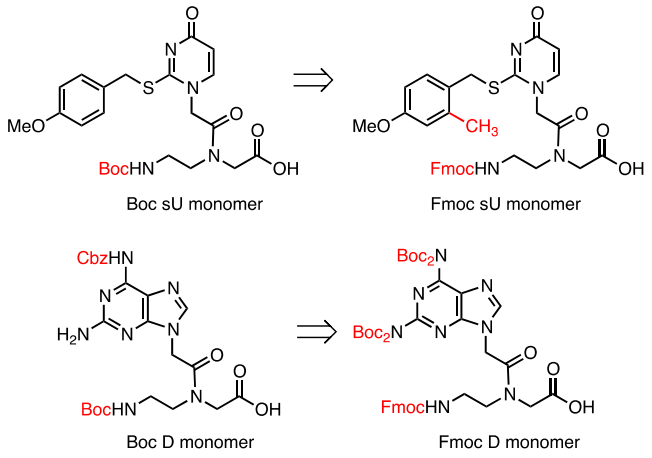


Fig. 2. Switching from Boc-based PNA monomers to Fmoc-based monomers.

monomers for pcPNA, the thiocarbonyl group of sU is protected with the MPM (*p*-methoxybenzyl) group and the exocyclic amino group of D is masked with the Cbz group. To remove these protecting groups from the nucleobases,harsh reagents such as trifluoromethanesulfonic acid (TFMSA) or HF are required. The main advantages of Fmoc chemistry are that after the completion of synthesis, PNA oligomers can be cleaved from the resin and fully deprotected under relatively mild acidic conditions (TFA) and that commercial peptide synthesizers typically employ Fmoc chemistry. Therefore, we selected the 4-methoxy-2-methybenzyl group (MMPM) to protect the thiocarbonyl group of the sU monomer for the following reasons: (1) Due to the electron-donating nature of the newly introduced methyl group, MMPM may be more sensitive to TFA than MPM and may be cleaved by a treatment with TFA; (2) MMPM may exhibit sufficient stability under the reaction conditions used for sU monomer synthesis; (3) since the 2-methyl group of MMPM is relatively small, its steric congestion may not affect the synthesis of the sU monomer and may not reduce coupling efficiency in the solid-phase synthesis of PNA oligomers. Regarding the D monomer, we decided to use the Boc group for amino protection. Here we report the development of new PNA monomers for pcPNA synthesis that are fully compatible with standard Fmoc chemistry (Fig. 2).

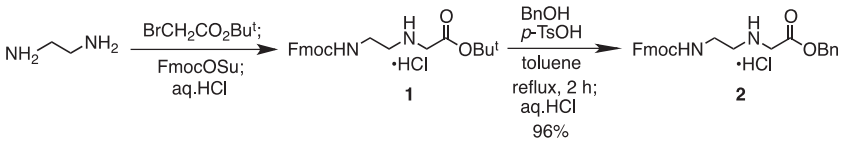
We selected benzyl *N*-[2-(Fmoc)aminoethyl]glycinate **2**[12] as the backbone unit for the synthesis of Fmoc-protected pcPNA monomers. The synthesis of **2** is outlined in Scheme 1. This scheme avoids the formation of 2-oxopiperazine, which has been occasionally observed in the synthesis of PNA backbones.[13,14] Compound **1** was prepared according to the procedure of Thomson et al.[15] **1** was then converted into benzyl ester **2** by a treatment with *p*-toluenesulfonic acid and the subsequent addition of excess benzyl alcohol in refluxing toluene. This one pot process proceeded efficiently and the requisite backbone **2** was obtained in excellent yield and on a large scale. Since **2** was isolated as a HCl salt, it was stable and could be stored for years at −20 °C.

The synthesis of Fmoc thiouracil monomer **11** commenced with the preparation of reagent **5** for protection of the thiocarbonyl group of 2-thiouracil (Scheme 2). The reduction of 2-methylanisaldehyde **3** with $NaBH_4$ gave alcohol **4**, which was converted to bromide **5** by a reaction with PBr_3. Since bromide **5** was not very stable, it was immediately used in the next step. The treatment of 2-thiouracil **6** with bromide **5** in a mixture of EtOH-H_2O gave S-alkylated derivative **7** in 88% yield. The alkylation of **7** with ethyl bromoacetate furnished a mixture of the N1- and O-alkylated regioisomers **8a** and **8b** in favor of the desired **8a**. Column chromatography provided **8a** and **8b** in 39% and 18% yields, respectively. This moderate yield of **8a** was acceptable because the precursor **7** was recovered in 38% yield and was reusable.

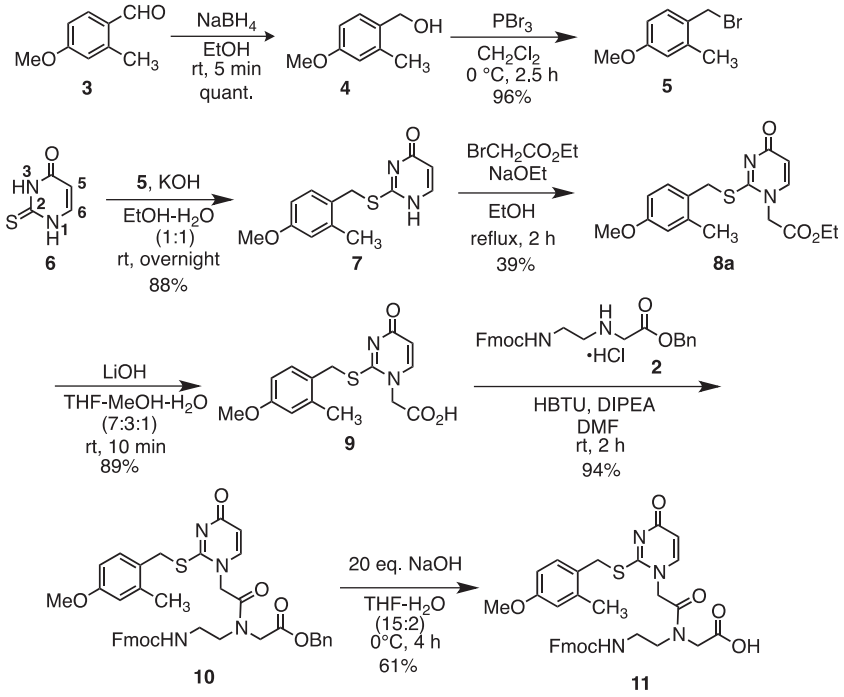
The alkylation of compound **7** may yield an N1-, N3-, or O-alkylated isomer (Fig. 3). The structure of **8a** was established as an N1-alkylated isomer through comparisons of its ^{1}H NMR and ^{13}C NMR spectra in DMSO-d_6[22] with those of the known structure.[5] The regiochemistry of **8a** was confirmed by NOE experiments. The presence of an NOE effect of the methylene protons of the –CH_2-COOEt fragment (δ 4.8 ppm) on the irradiation of the pyrimidine H-6 (δ 7.7 ppm) was indicative of an N1-alkylated isomer. Additional evidence was obtained from the HMBC experiment for **8a** in which methylene protons were coupled to *C*2 and C6 carbons (red arrows in Fig. 3).

The regiochemical assignment of **8b** was performed as follows. Although compound **7** may be alkylated at the N3 position, this possibility was excluded by comparisons with the reported NMR data of the S- and N3-dialkylated isomers of thiouracil.[16,17] Furthermore, the HMBC experiment for **8b** showed that the methylene protons of the –CH_2-COOEt fragment (δ 5.0 ppm) were not coupled to *C*2 carbon. Since this coupling is expected for an N3-isomer, the possibility of alkylation at the N3 position was again discounted. Therefore, the remaining possibility was an O-alkyl isomer. The ^{13}C chemical shift of the methylene carbon of **8b** (δ 62.7 ppm) in DMSO-d_6 was shifted downfield from that of the analogous carbon of **8a** (δ 52.4 ppm),[22] which indicated that the carbon atom of the methylene linker (-CH_2-) directly bonded to an oxygen atom. In addition, the ^{1}H NMR and ^{13}C NMR spectra of **8b** in $CDCl_3$ were consistent with those reported for the S- and O-dialkylated isomers.[16–18] Thus, **8b** was an O-alkylated isomer.

The ethyl ester **8a** was saponified with LiOH to afford carboxylic acid **9** in 89% yield. The coupling of **9** with PNA backbone **2** using HBTU provided **10** in 94% yield. The benzyl ester group of **10** was selectively cleaved by an alkaline treatment (20 eq. NaOH in



Scheme 1. Synthesis of Fmoc-protected PNA backbone unit.



Scheme 2. Synthesis of Fmoc-protected thiouracil monomer.

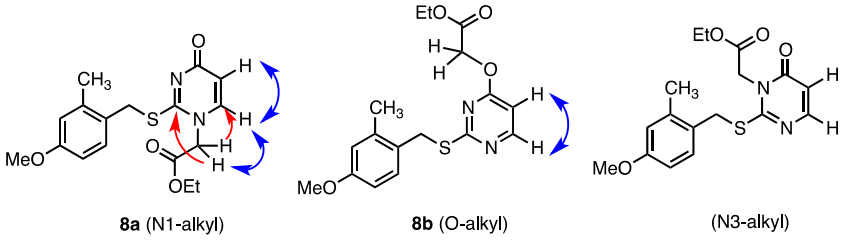
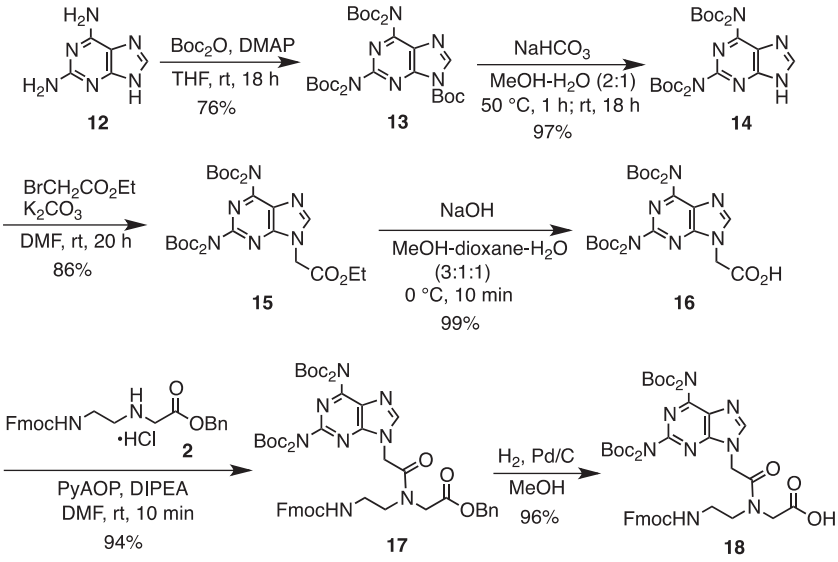


Fig. 3. Possible structures of alkylation products of **7**. Selected NOE correlations (blue double-headed arrows) and HMBC H-C correlations (red arrows) of compounds **8a**, **8b**.

THF)[19] to afford sU PNA monomer **11** in 61% yield. Fortunately, removal of the base-labile Fmoc group was negligible under this condition (Scheme 2).

Fmoc/Boc-protected diaminopurine monomer **18** was synthesized by adapting the procedure reported by Hudson et al.[20] with some modifications (Scheme 3). The treatment of commercially available 2,6-diaminopurine **12** with an 8 M equiv. of Boc_2O and catalytic amount of DMAP in THF gave penta-Boc diaminopurine **13** in 76% yield. Compound **13** was converted to tetra-Boc diaminopurine **14** under the conditions previously established for the mono-Boc deprotection of Tris-Boc adenine in high yield (97%).[21] Alkylation of the N9 position of **14** with ethyl bromoacetate provided compound **15** (86%), which was subjected to saponification of the ethyl ester group by a treatment with aqueous NaOH in MeOH/dioxane to afford **16** in excellent yield. Due to the poor solubility of **15** in MeOH, dioxane was added as a co-solvent. The coupling of **16** with the backbone **2** was accomplished with PyAOP in DMF, giving benzyl ester **17** in 94% yield. The catalytic hydrogenation of **17** gave Fmoc-protected diaminopurine monomer **18** in 96% yield.

To test the compatibility of the newly synthesized PNA monomers with standard Fmoc-based solid-phase synthesis,[22] D and sU monomers were incorporated into a 10-residue mixed-base PNA sequence, ***P1***, H-*Lys*-GsUDGDsUCDCsU-*Lys*-NH_2. The oligomer ***P1*** was manually synthesized on a 8 μmol scale using a 2.5 M equiv. of the monomers and 1.7 M equiv. of HATU as a coupling agent. Regarding G and C, commercially available Fmoc/Bhoc-protected monomers were used. After the completion of oligomerization, the resins were treated with TFA containing 13.9% *m*-cresol and 2.8% H_2O for deprotection and cleavage from the resins. The



Scheme 3. Synthesis of Fmoc-protected diaminopurine monomer.

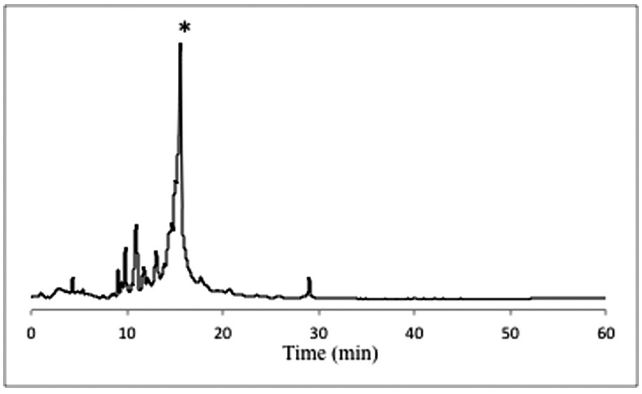


Fig. 4. Reversed-phase HPLC analysis of crude oligomer. The peak that corresponds to the expected 10-mer PNA (**P1**, H-*Lys*-GsUDGDsUCDCsU-*Lys*-NH_2) is marked by an asterisk.

HPLC and MALDI-TOF MS analyses indicated that there was no remaining MMPM on the sU nucleobase (Fig. 4). The PNA oligomer was purified by HPLC and characterized by mass spectrometry (average mass from MALDI-TOF MS and exact mass from ESI-Orbitrap MSFig. 5). These results demonstrated that the MMPM used for thiocarbonyl protection was intact during PNA oligomerization and cleanly removed by the TFA treatment.

In conclusion, we synthesized Fmoc-protected sU and D PNA monomers and incorporated them into a well-documented 10-mer PNA oligomer.[5] The newly synthesized monomers were fully compatible with standard Fmoc-based solid-phase synthesis. In the Boc-based sU monomer, MPM has been used for thiocarbonyl protection and this group is resistant to TFA. Regarding the Fmoc-based sU monomer, we selected MMPM instead of MPM. MMPM on sU was found to be more acid sensitive than MPM and was easily deprotected by the TFA treatment.

The results of the present study will contribute to improvements in practical Fmoc-based pcPNA synthesis. Fmoc chemistry may avoid harsh reagents, and, thus, is favorable for researchers, particularly for biologists. Easy access to pcPNAs will facilitate their applications, such as antigene PNAs and DNA nanotechnologies.

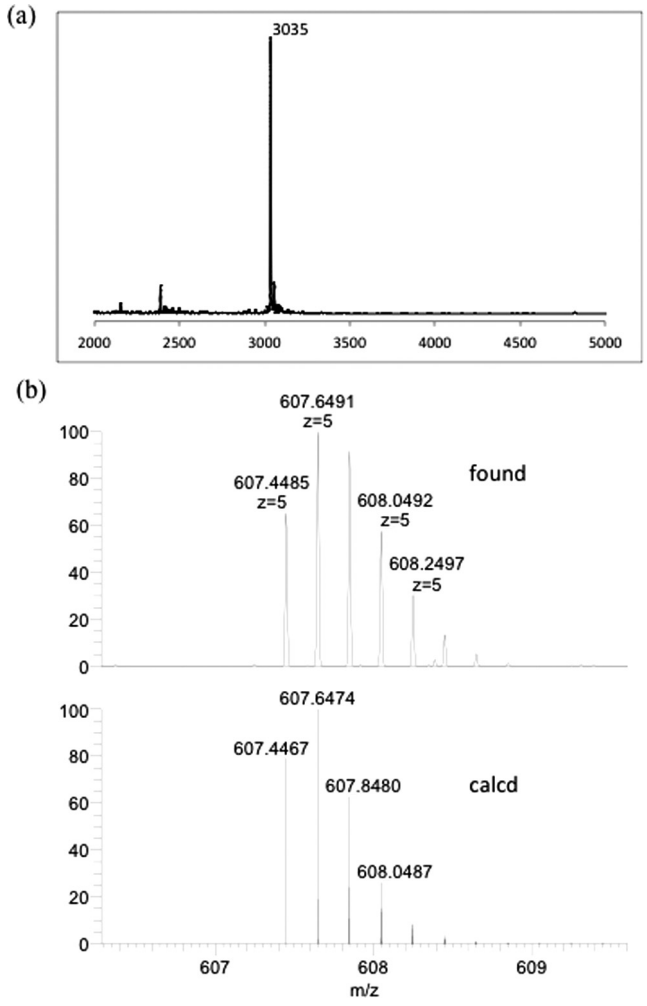


Fig. 5. (a) MALDI-TOF mass spectrum of the purified 10-mer **P1**. $[M + H]^+$ calcd for $C_{117}H_{158}N_{65}O_{29}S_3$ = 3035, found 3035. (b) ESI-Orbitrap mass spectrum of **P1**. $[M + 5H]^{5+}$ calcd for $C_{117}H_{162}N_{65}O_{29}S_3$ = 607.4467, found 607.4485.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.06.015.

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