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PNA/DNA interstrand cross-links from a modified PNA base upon photolysis or oxidative conditions

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ABSTRACT

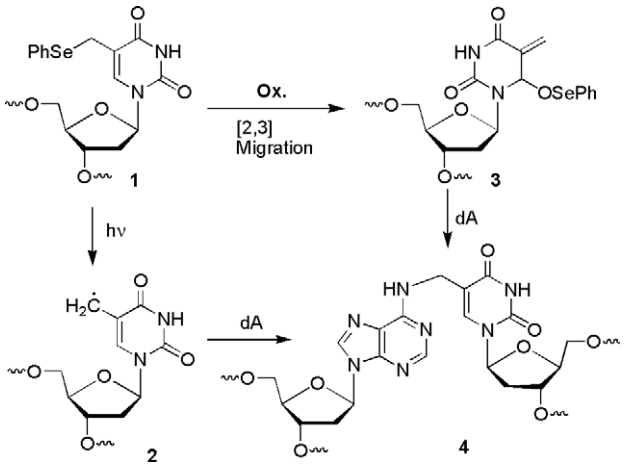
PNA/DNA interstrand cross-links (ICLs) were observed when peptide nucleic acids (PNAs) containing modified thymine derivatives were hybridized with the complementary or one-base mismatched DNA upon photolysis or treatments of oxidative agent. PNA/DNA ICL formation provides a useful method for biological applications such as antisense technologies or PNA chips.

Peptide nucleic acids (PNAs)[1] have widespread applications in many areas such as bio-drug for antigene/antisense therapy and molecular tools for molecular biology and functional genomics.[2] Many of theses applications involve the hybridization of PNA oligomers to DNA targets to form PNA/DNA duplexes because of the characteristics of strong PNA/DNA binding modes. If further treatments of these PNA/DNA duplexes such as UV-photolysis or oxidants can produce a covalent linkage between a PNA and a DNA strand, covalently linked PNA/DNA duplexes could become an inert molecule permanently under thermodynamic conditions.

Recently, DNA/DNA interstrand cross-links (ICLs) formation from a modified nucleotide was first reported by the Greenberg group.[3] ICLs formation in duplex DNA after exposure to γ-radiolysis is a particularly exciting result because the modified nucleotide triphosphate could be a useful radiosensitizing agent.[4]

The Greenberg group showed that a DNA duplex containing a phenyl selenide derivative (**1**) produces DNA interstrand cross-links with the opposing strand dA upon photolysis or oxidative conditions (Scheme 1). Cross-linking proceeds through the 5-(2′-deoxyuridinyl) methyl radical (**2**) upon photolysis[3] or methide (**3**) under oxidative conditions.[5] Both reaction conditions produce a kinetic ICL product believed to be a dA-N1 adduct, which isomerizes in solution to the stable dA-N6 adduct (**4**) by Dimroth rearrangement.[6]

Herein, we describe the possibility that ICL formation can be extended to a PNA/DNA duplex using a PNA probe containing phenyl selenide-modified thymine (**6**). If PNA/DNA ICLs are formed using a modified PNA probe when treated with photolysis or oxidative stress, they could be a useful tool for PNA biotechnological applications such as antisense techniques and PNA chips.



Scheme 1. Proposed mechanisms of DNA ICL formation from a modified nucleotide upon photolysis or oxidative stress.[6]

A phenyl selenide-modified thymine 1-acetic acid (**6**) was prepared from thymine 1-acetic acid methyl ester (**5**) by modifying the previously reported procedure for thymidine derivative[7] (Scheme 2).

First, the photochemical activity of phenyl selenide derivative (**6**) was determined under aerobic conditions. After the photolysis

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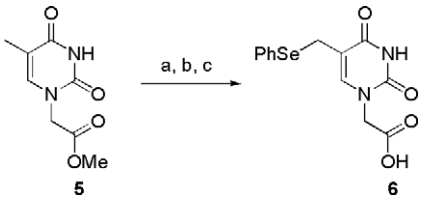


Scheme 2. Reagents and conditions: (a) NBS, benzene, AIBN; (b) PhSe-SePh, $NaBH_4$, DMF, 52% (two steps); (c) aq NaOH/Dioxane, 98%.

of **6** at 350 nm, the photolysates were analyzed by HPLC. Consumption of **6** was monitored using an internal standard marker (thymidine, **T**) by reverse phase HPLC (Fig. 1). Compound **6** (1.0 mM) was consumed to about 90% within 30 min.

Upon photolysis, the intermediate expected could be a 5-(1-carboxymethyl-uracil) methyl radical (**7**) like the nucleoside analogue (**2**). We believe that several stable products that eluted out faster than the internal standard are derived from the peroxy radical **8**, as proposed previously, and/or the reduction of the hydroperoxide (Scheme 3).[7,8]

Next, we tested that oxidative stress can produce a reactive methide, which is the intermediate for the ICL formation in DNA under oxidative conditions. We confirmed via NMR experiments that a reactive methide intermediate (**10**) is generated under oxidative conditions ($NaIO_4$ treatment).

Phenyl selenide thymine derivative (**6**) was oxidized into selenoxide (**9**) by $NaIO_4$ in good yield, and allylic selenoxide underwent [2,3]-sigmatropic rearrangements to produce a methide (**10**) like nucleoside analogue **3**, as is shown in Figure 2. The production of methide intermediate was confirmed by the appearance of vinyl protons (H_A and H_B in Fig. 2C). Finally, 5-hydroxymethyluracil 1-acetic acid **11** was produced through a reaction with water during the overnight incubation.

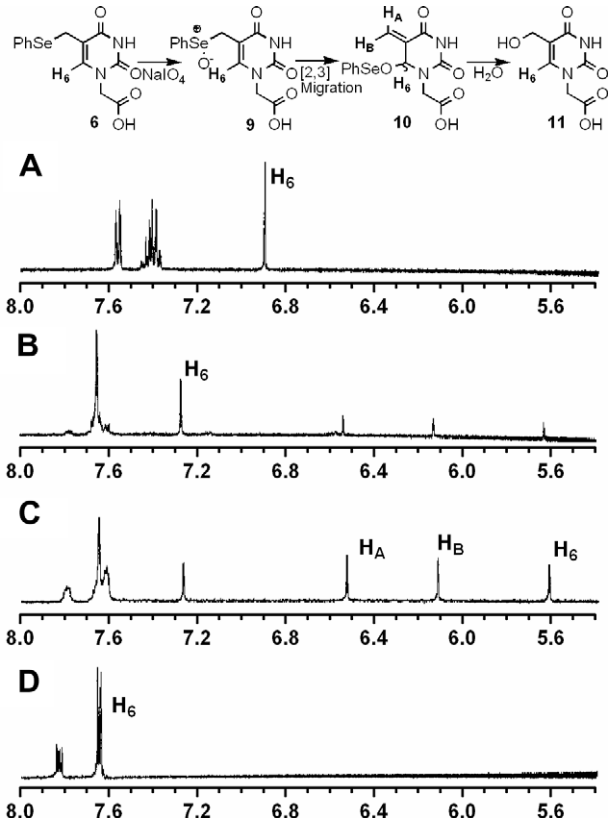


Figure 2. ^1H NMR analysis of the reaction of **6** (1 mM) with $NaIO_4$ (2 mM) in deuterated phosphate buffer (10 mM, pD 7.0). (A) Before $NaIO_4$ addition; **6**, (B) 10 min after $NaIO_4$ addition; **9**, (C) 2 h after $NaIO_4$ addition; **10**, and (D) 16 h after $NaIO_4$ addition; **11**.

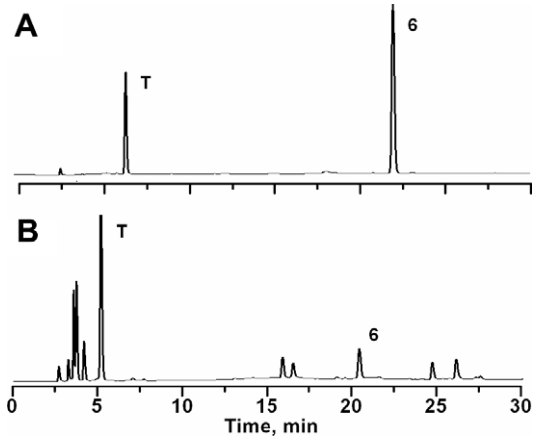
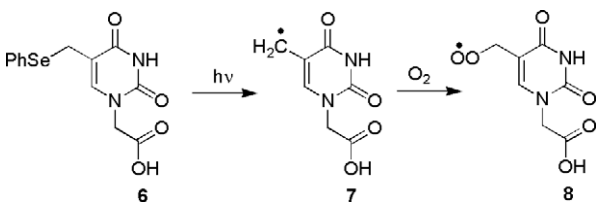


Figure 1. HPLC analysis of the photolysates of **6**. (A) Before photolysis, (B) after photolysis at 350 nm for 30 min, internal standard; thymidine (**T**).



Scheme 3. Methyl radical intermediate upon the photolysis of **6**.

The rate constant of the formation of intermediate **10** was determined by NMR experiments using an internal standard (thymidine). The disappearance of the H_6 hydrogen of **9** was monitored in time courses (Fig. 3). The plotting was well fixed to the first-order rate equation. However, phenyl selenoxide derivative **9** ($k_0 = 5.30 \times 10^{-4}\ s^{-1}$, $t_{1/2} = 21.8$ min) rearranged approximately ten times more slowly than the comparable nucleoside thymidine derivative ($6.1 \times 10^{-3}\ s^{-1}$, $t_{1/2} = 1.9$ min).[9] Hence, we confirmed that the reactivity of phenyl selenide **6** is similar to that of nucleoside analogue **1** upon photolysis or oxidative conditions.

Precursor **6** was independently introduced at the defined site in PNA oligomer (**12**) using standard Fmoc-solid phase synthesis

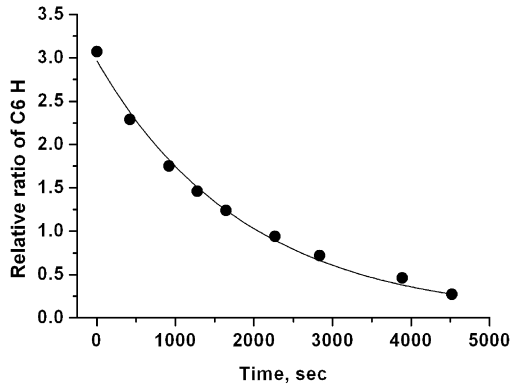


Figure 3. The rate constant determination of the formation of methide intermediate **10**.

methods.[10] The N-terminal of PNA probe was modified with the acetyl group (Scheme 4).

DNA oligomers were purchased from Bioneer Inc. DNA**13** is a fully matched 15 mer to the modified PNA (**12**) and DNA**14** is a mismatched sequence in the base opposite of the precursor site in PNA (**12**). DNA**15**–**17** were designed to determine the mismatched sequence effects.

In order to determine the ICLs formation between the PNA probe and DNAs, 5′-fluorescein labeled DNAs were hybridized with a PNA probe under standard hybridization conditions, and then PNA/DNA duplexes were further treated with photolysis or $NaIO_4$.[11] Denaturing polyacrylamide gel electrophoresis analysis reveals the formation of products whose migration is severely retarded relatively to unreacted DNA oligomers, indicative of interstrand cross-linked products (Fig. 4).

In photolysis, ICL was formed in significant quantities in a fully matched PNA/DNA**13** duplex (Fig. 4A), but the formation of ICLs in single-base mismatched sequences (PNA/DNA**14**–**17**) was less than 10% yield except that of PNA/DNA**15**. This is consistent with the previous results from DNA/DNA ICL formation.[4] Under oxidative stress, the extents of ICL formation were dramatically changed. A fully matched PNA/DNA**13** duplex with treatment of $NaIO_4$ did not produce significant interstrand cross-link. Also, ICL formation in distal mismatched sequences (DNA**16** and **17**) was negligible. However, ICL formation was accelerated in an opposing base or adjacent base-mismatched sequence PNA/DNA**14**, PNA/DNA**15**, respectively (Fig. 4B).

A characterization of the cross-linked product from the treatment with $NaIO_4$ of PNA/DNA**15** was obtained by MALDI-TOF mass.[12] The observed molecular weight (calculated m/z 8666.9; found m/z 8664.8) is well consistent with that of the reaction between **6** and the opposing DNA strand (Fig. 5). The observed molecular weight is also consistent with the observation that O_2 is unnecessary for interstrand cross-link formation as in the DNA ICLs formation.

In contrast to ICL formations of photolysis, which are produced from the radical pathway, interstrand cross-links of modified PNA/DNA under oxidative stress are formed from the methide type intermediate (**10**). Further support for this mechanism was obtained by determining the rate constant for ICL formation in

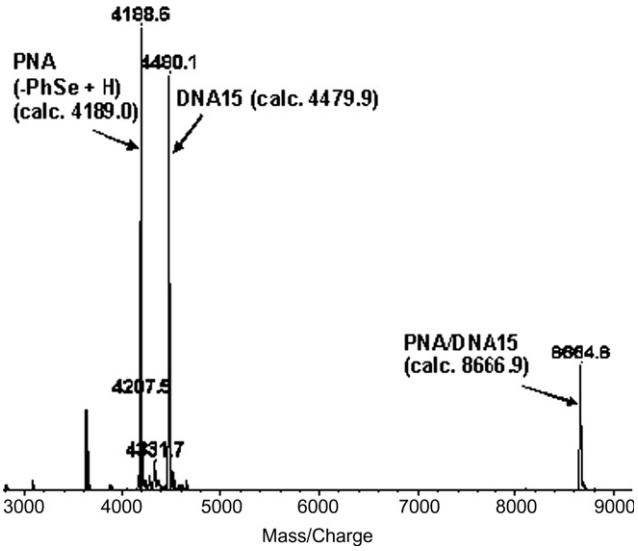


Figure 5. MALDI-TOF mass of the cross-linked PNA/DNA obtained upon $NaIO_4$ treatment of PNA/DNA**15**.

PNA/DNA**15** treated with $NaIO_4$ (5 mM) (Fig. 6A). Phenyl selenoxide derivative **9** ($t_{1/2}$ = 21.8 min) rearranged approximately 10 times more slowly than the comparable nucleoside thymidine derivative ($t_{1/2}$ = 1.9 min).[9] Hence, the plotting was carried out at 20 min as an initial background after the treatment of $NaIO_4$. ICL growth was well fixed first-order kinetics (Fig. 6B). The observed rate constant for ICL formation ($6.9 \times 10^{-4}\ s^{-1}$, $t_{1/2}$ = 16.9 min) was comparable to that of DNA ICL formation ($t_{1/2}$ = 28.2 min).

In contrast to the reactivity of the thymidine analogue (**1**), we found that the fully matched PNA/DNA**13** under oxidative stress did not produce a significant amount of ICLs as shown in Figure 4B. It can be explained by local duplex stability. The effect of the phenyl selenide group on duplex thermal stability was analyzed by measuring the UV-melting temperature (T_m, Table 1). The phenyl selenide group reduced the T_m by 0.8 °C relative to when thy-

PNA probe (**12**): GTA GTA G**6**T ATA TTG-*N*-Ac
PNA control (**18**): GTA GTA G**T**T ATA TTG-*N*-Ac
DNA **13**: 5'-F- CAT CAT C**A**A TAT AAC-3'
14: 5'-F- CAT CAT C**C**A TAT AAC-3'
15: 5'-F- CAT CAT CA**C** TAT AAC-3'
16: 5'-F- CAT CAT CAA **G**AT AAC-3'
17: 5'-F- CAT CAT CAA T**C**T AAC-3'

Scheme 4. The sequences of a modified PNA probe, fully matched (DNA**13**) and single-base mismatched DNA oligomers (DNA**14**–**17**). 5′ sites of DNAs were labeled with fluorescein.

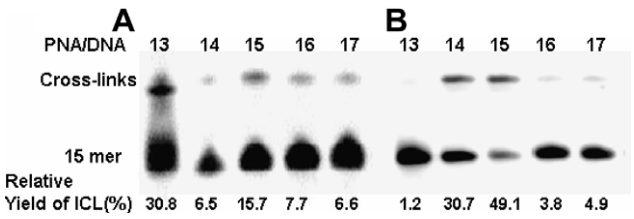


Figure 4. Denaturing PAGE analysis of PNA/DNA duplexes. (A) Photolysis (350 nm, 30 min), (B) $NaIO_4$ treatments (5.0 mM, 37 °C for 2 h).

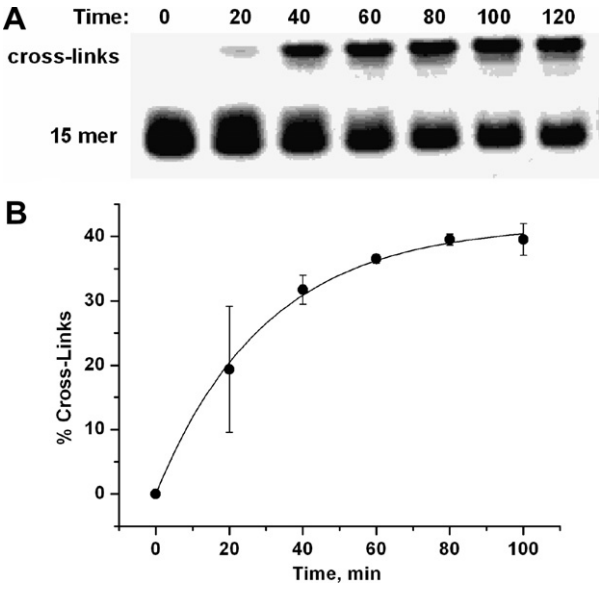


Figure 6. Rate of ICL growth from PNA/DNA**15** upon $NaIO_4$ (5 mM, 37 °C) treatments. (A) Denaturing PAGE analysis, (B) ICL growth follows first-order kinetics.

Table 1
Comparison of the UV-melting temperature of PNA/DNA duplexes containing modified **6**

PNA	DNA[a]	T_m (°C)[b]
18	13	63.0 ± 0.0
12	13	62.2 ± 0.5
12	14	49.4 ± 0.5
12	15	49.0 ± 0.1
12	16	50.3 ± 0.8
12	17	52.8 ± 0.7

[a] Sequences of DNA were the same as those of DNA**13**–**17**, but without labeling of fluorescein.
[b] [PNA], [DNA] = 1.0 μM, phosphate buffer (20 mM, pH 7.2).

mine was present. The effect of a mispair opposite **6** was similar to when one mispair was present in other sites.

Recently, the Greenberg group described flanking sequence effects on cross-linking reactions in a DNA duplex containing a phenyl selenide-modified 5-methyl-deoxycytidine.[9] Almost no ICL formation in fully matched PNA/DNA**13** and distal single-base mismatched sequences (PNA/DNA**16**, PNA/DNA**17**) indicates that the local duplex stability could be the cause of the sequence effect on cross-linking because **6** must adopt the *syn*-conformation in order to react with the opposing DNA strand.

The methide type intermediate **10** cannot adopt the *syn*-conformation in a stable local duplex environment. Hence, no ICL formations were observed in fully matched and distal single-base mismatched DNA sequences. However, there was a high yield of ICL formation in a mispair opposite **6** and adjacent mispair sequence due to more flexible local stability. A cross-linking site opposite of DNA**14** strand in the PNA/DNA**14** duplex might be a 3′-adjacent dA, which was reported by the Greenberg group.[9]

In summary, PNA/DNA interstrand cross-links were formed from modified PNA base upon photolysis or oxidative conditions. In contrast to photolysis, ICLs formation in $NaIO_4$ treatment was observed in mismatched DNA sequences in the vicinity of opposing **6**. PNA/DNA ICL formation could be a useful tool for PNA biotechnological applications such as PNA chips and PCR clamping.

Acknowledgments

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10. PNAs were provided from the Panagene Inc. MALDI-TOF-mass: PNA**12**, calcd 4344.0 (M), found: 4345.1 (M+1); PNA**18**, calcd 4189.0 (M), found: 4189.8 (M+1).
11. Hybridization conditions: [PNA] = 20 μM, [DNA] = 10 μM, 10 mM, pH 7.2, phosphate buffer, incubated at 90 °C for 5 min, and then slowly cooled to room temperature. This is followed by further treatments (5 mM $NaIO_4$ or 350 nm photolysis).
12. Samples for MALDI-TOF mass were pretreated with the C18-Sepak column to remove salts in reaction solutions.