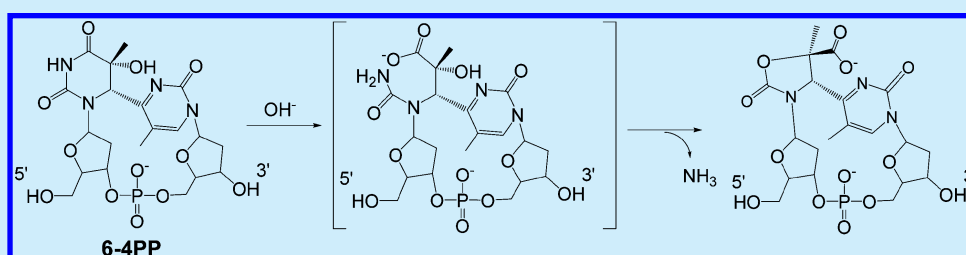




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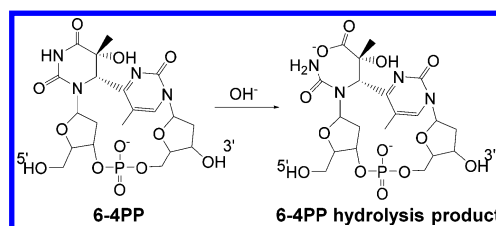
An Unexpected Deamination Reaction after Hydrolysis of the Pyrimidine (6-4) Pyrimidone Photoproduct

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ABSTRACT: Pyrimidine (6-4) pyrimidone photoproduct (6-4PP), a common DNA photolesion formed under solar irradiation, was indicated to hydrolyze under strong basic conditions, breaking the N3–C4 bond at the 5'-thymine. The reanalysis of this reaction revealed that the resulting water adduct may not be stable as previously proposed; it readily undergoes an esterification reaction induced by the 5-OH group at 6-4PP to form a five-membered ring, eliminating a molecule of ammonia.

Pyrimidine (6-4) pyrimidone photoproduct (6-4PP) is a common DNA photodamage product resulting from the UV component of sunlight. Its presence leads to a sharp kink, which significantly distorts the DNA helical structure.¹ In contrast, another common pyrimidine photoproduct, the cyclobutane pyrimidine photoproduct (CPD), causes relatively minor structural disturbance, e.g. a 30° helical bend toward the major groove.² 6-4PP is much more mutagenic than CPD.^{3–5} It arrests the replication forks of general replicative DNA polymerases.⁶ When bypassed by the human Y-polymerase pol η , there is a 7-fold tendency for a G instead of an A to be inserted into the position opposite to the 3'-T of the 6-4PP lesion.^{3,7,8} Due to the resulting drastic DNA structural change, 6-4PP is readily recognized by the DNA repair machinery, resulting in a quick removal in living cells (half-life of 6-4PP = 2.3 h).⁹ The unrepaired 6-4PP is suggested to be the primary cause of UVB-induced cell apoptosis especially in NER-deficient cells.^{10,11}

It is thus of significance to understand the physical properties of 6-4PP. 6-4PP is shown to be alkaline labile, inducing DNA strand scission upon hot alkaline treatment.^{12–15} Research from the Iwai laboratory suggests that the alkaline treatment first ruptures the N3–C4 bond on the 5'-thymine of 6-4PP to yield a hydrolysis product (Scheme 1),¹⁶ which is followed by a deglycosylation reaction at the 3'-thymine,^{15,17} leading to an ultimate DNA strand scission. Recently, our group proved that the third naturally occurring thymine dimer, i.e. 5-thyminyl-5,6-dihydrothymine which is commonly referred to as the spore

Scheme 1. Previously Indicated Hydrolysis Reaction of 6-4PP

photoproduct (SP), and 5,6-dihydro-2'-deoxyuridine (dHdU) resulting from the ionizing radiation damage to cytosine under anoxic conditions^{18,19} also undergo the N3–C4 bond rupture via a hemiaminal intermediate, suggesting that such a bond cleavage is probably a common feature possessed by a saturated pyrimidine residue.²⁰ Our re-examination of the 6-4PP decomposition reaction however implies that the resulting water adduct is not stable as previously suggested;¹⁶ it undergoes an additional deamination reaction to yield 2-oxazolidinone (5-4) pyrimidone, eliminating the N3 as an ammonia. This report brings new insight into the reactivity of 6-4PP, which is important toward ongoing synthetic, analytical, and biological studies, i.e., handling and generating DNA containing 6-4PP and the quantitation of 6-4PP in DNA, and

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thus may provide a fundamental understanding of the biological consequences of this common DNA photolesion.

We obtained 6-4PP via irradiation of the dinucleotide TpT in acetonitrile under 254 nm UV light using a published protocol.²¹ The 6-4PP was isolated by HPLC and redissolved in 50 mM KOH to a final concentration of 0.75 mM. The resulting solution was heated to 60 °C for various times and analyzed by HPLC. Such a reaction condition is similar to that adopted in the previous 6-4PP hydrolysis studies.¹⁶ As shown in Figure 1, compound **1** was isolated after a 5-h reaction as the

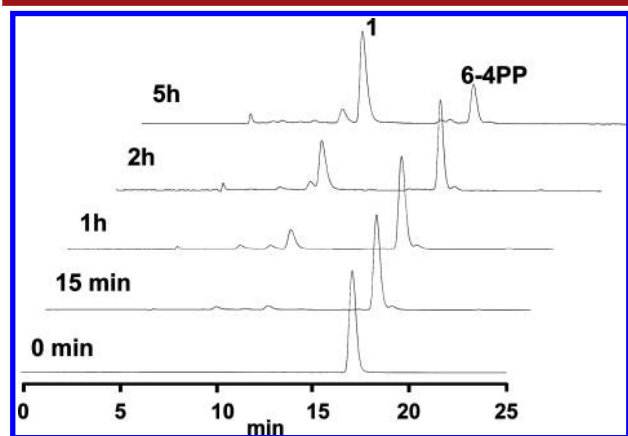


Figure 1. HPLC chromatograph of the 6-4PP hydrolysis reaction in 50 mM KOH at 60 °C monitored by UV detector at 310 nm. The reaction affords **1** as the major product. None of the minor products exhibits a molecular mass corresponding to that of the 6-4PP water adduct as indicated by the ESI-MS analysis.

dominant product (~70% yield). However, to our surprise, ESI-MS analysis under the negative ion mode found that the $[M - H]^-$ species of **1** exhibits an m/z signal of 546.12 amu, which is +1 amu higher than that of 6-4PP, but is -17 amu smaller than the predicted 6-4PP water adduct shown in Scheme 1 (563.14).¹⁶ None of the minor reaction products exhibits an $[M - H]^-$ signal of 563.14 either. If the reaction was conducted in 0.2 M KOH at room temperature, a cleaner formation of **1** was observed while the reaction pattern remained the same.²²

The formation of **1** was observed from the very beginning of the reaction; no reaction intermediate en route to **1** was detected in our hands. Because the previous studies reported that 6-4PP was hydrolyzed under strong basic conditions, we wonder whether **1** derives from this hydrolysis reaction. We therefore carried out the reaction in 0.2 M KOH dissolved in 97% ¹⁸O-labeled water. The same product was generated as indicated by the HPLC analysis. ESI-MS analysis reveals an m/z signal of 548.12 in the $[M - H]^-$ form,²² which has +2 amu relative to that of **1** produced in unlabeled water. This observation is in line with the above hypothesis, indicating that **1** indeed results from the 6-4PP hydrolysis reaction. However, again, the mass is reduced by 17 amu compared with the predicted mass for the 6-4PP water adduct with one ¹⁸O atom incorporated.

Typically, a mass of 17 amu corresponds to an ammonia molecule; we thus wonder whether the amino moiety attached to the C2=O at the 5'-thymine of the hydrolysis product has been removed. To test this hypothesis, we first incorporated a ¹⁵N label at the N3 position via a N-nitration reaction of the uridine residue²³ and synthesized a dinucleotide TpT with the

5'-thymine containing the ¹⁵N label. The corresponding 6-4PP was then prepared photochemically as described above. The labeled 6-4PP exhibits an $[M - H]^-$ signal of 546.13, and a single peak at 191.7 ppm in the ¹⁵N NMR spectrum (Figure 2).

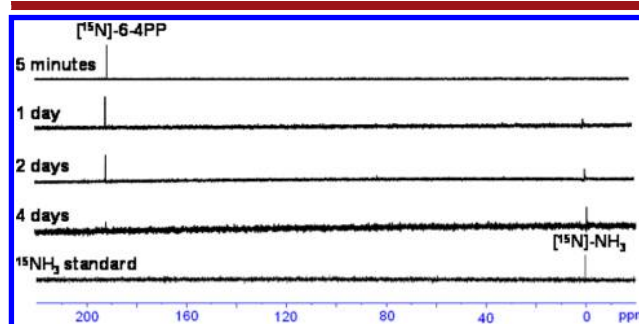


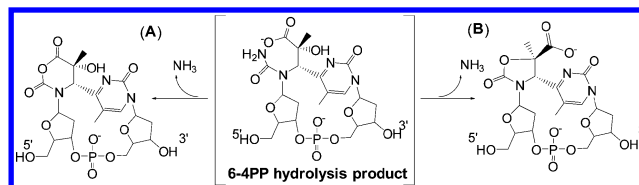
Figure 2. ¹⁵N NMR spectra describing the deamination reaction using ¹⁵N labeled 6-4PP in 0.30 M KOD at ambient temperature in D₂O. The ¹⁵N peak (191.7 ppm), from the ¹⁵N moiety in 6-4PP, decreased over time as the reaction proceeded. A new ¹⁵N peak at 0 ppm, corresponding to ¹⁵NH₃, increased correspondingly, indicating that a molecule of ammonia was released during the reaction.

Treatment of the resulting [¹⁵N]-6-4PP with 0.2 M KOH at ambient temperature again affords **1**. ESI-MS analysis of the newly formed **1** reveals an $[M - H]^-$ signal of 546.11, which is the same as that generated from unlabeled 6-4PP, supporting the assumption that the N3 moiety is eliminated. Additionally, the m/z signal of **1** is similar to that of the [¹⁵N]-6-4PP. The small mass difference (~0.02 amu) due to the involvement of various isotopes can be readily resolved by high-resolution mass spectrometry.²² This observation further suggests that **1** is formed by hydrolysis of 6-4PP (+ 18 amu due to the added water) followed by loss of an ammonia (- 18 amu due to the loss of ¹⁵NH₃).

The ¹⁵N label provides a marker for NMR analysis, enabling us to follow the 6-4PP hydrolysis reaction via NMR spectroscopy. During the 4 days of reaction in 0.3 M KOD, a gradual loss of the ¹⁵N signal at 191.7 ppm was observed, in agreement with the decomposition of 6-4PP. This signal loss is accompanied by the growth of a new ¹⁵N peak at 0 ppm (Figure 2). This new signal is ascribed to the released ¹⁵NH₃, as confirmed using commercially available ¹⁵NH₃ standard.

The 6-4PP hydrolysis reaction was suggested to rupture the N3-C4 bond, resulting in a stable water adduct (Scheme 2).¹⁶

Scheme 2. Two Possible Deamination Mechanisms



Although we were unable to observe this product during our 6-4PP hydrolysis study, its formation is highly likely as indicated by the fully characterized similar hydrolysis reactions in alkaline treated SP and dHdU.²⁰ It is thus of interest to reveal how this 6-4PP water adduct deaminates. Two possible mechanisms, via formation of an anhydride (Scheme 2A) and via an esterification reaction involving the 5-OH group at the 5'-thymine of 6-4PP (Scheme 2B), can be proposed. Although

similar anhydride species were observed in the MS/MS analyses of the SP hydrolysis reaction,²⁰ and of the oxanosine deamination process,²⁴ such a compound is not stable in aqueous solution, as indicated by the slow decomposition of uracil anhydride in ethanol at room temperature.²⁵ Moreover, considering the similar structures among the hydrolysis products of SP, 6-4PP, and dHdU, if route A is possible, deamination processes should be observed during the hydrolysis of SP and dHdU. The lack of anhydride formation in these cases²⁰ indicates that the esterification mechanism shown in route B is more likely.

This conclusion is further supported by the NMR characterizations of **1** as detailed below:

- (1) The $-\text{CH}_3$ and H6 atom on the 5'-thymine of 6-4PP adopt a *cis* configuration, resulting in a strong ROE signal.²² Such an interaction is lost in the newly formed **1**, which is consistent with the formation of a five-membered ester ring via route B, where the $-\text{CH}_3$ is *trans* to the H6.
- (2) Comparing the ^{13}C NMR spectrum of 6-4PP with that of **1** indicates that very minor changes on chemical shift were observed in both 2'-deoxyriboses and the 3'-thymine ring, while relatively large changes were observed on carbons at the 5'-thymine ring (Table S1).²² Particularly, chemical shifts of -6.8 and 10.9 ppm were observed for its methyl group and C5 respectively, indicating drastic structural changes associated with these groups. Such changes are obvious in the ester product, but not so in the putative anhydride species.
- (3) ^{18}O isotope shifts in ^{13}C NMR spectroscopy have been utilized to facilitate chemical structure determination.^{24,26–30} We therefore mixed ^{18}O labeled **1**, prepared by 6-4PP hydrolysis in ^{18}O -labeled water,²² with unlabeled **1**. Using ESI-MS analysis, the ratio between the unlabeled and ^{18}O labeled **1** was found to be 1:1.8.²² We then acquired the ^{13}C NMR spectrum for this compound mixture. In the ^{13}C NMR spectrum, the C4 signal exhibits an upfield shift of 29 ppb which is in line with the isotope shifts observed in other ^{18}O substituted carboxylates;³¹ the C2 signal still exists as a single peak (Figure 3). This observation suggests that only C4 is connected to an ^{18}O (route B). If the anhydride is formed, both C2 and C4 signals are expected to exhibit an isotope shift.

Taken together, our data suggest that the 6-4PP hydrolysis product may not be stable as previously assumed; it undergoes an additional deamination process driven by esterification with the 5-OH at 5'-thymine to form a five-membered ring, resulting in 2-oxazolidinone (5-4) pyrimidone. Rupture of the N3–C4 bond was also observed in our recent SP and dHdU hydrolysis studies,²⁰ resulting in stable water adducts. Different from SP and dHdU, 6-4PP has an extra 5-OH moiety leading to the unexpected deamination reaction reported here. The five-membered ring is considered to be the most kinetically favorable configuration in ring closing reactions due to the lowest entropic cost and transition state strain energy.^{32,33} This kinetically favorable ring closure process determines that the formed 6-4PP water adduct must be very short-lived. Considering the heterocyclic ring structures of nucleobases, similar deamination reactions may occur after ring opening in certain nucleobase modifications, which await further studies in the future.

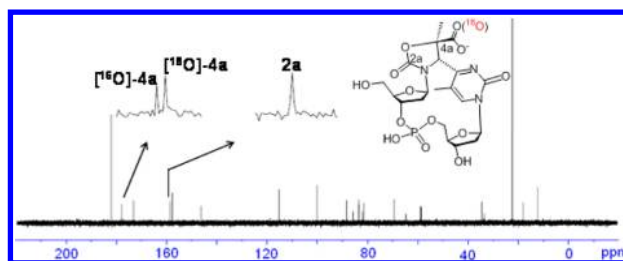


Figure 3. ^{13}C NMR spectrum using a mixture of **1** generated from hydrolysis of 6-4PP at the presence of 0.2 M KOH in unlabeled and ^{18}O labeled water, respectively. The ratio between unlabeled (^{16}O) and ^{18}O labeled **1** is suggested to be 1:1.8 by the ESI-MS analysis. An upfield isotope shift of 29 ppb was observed for C4a, and no shift was observed for C2a in the ^{13}C NMR spectrum. This result suggests that the ^{18}O atom is attached to C4a, but not to C2a, as indicated by the Chemical structure included. Integration of the two C4a signals reveals a ratio of 1:1.71, agreeing with the ratio revealed by the ESI-MS analysis and supporting the signal assignment shown in the figure.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis of the 6-4PP, NMR characterization of the reaction products. 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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