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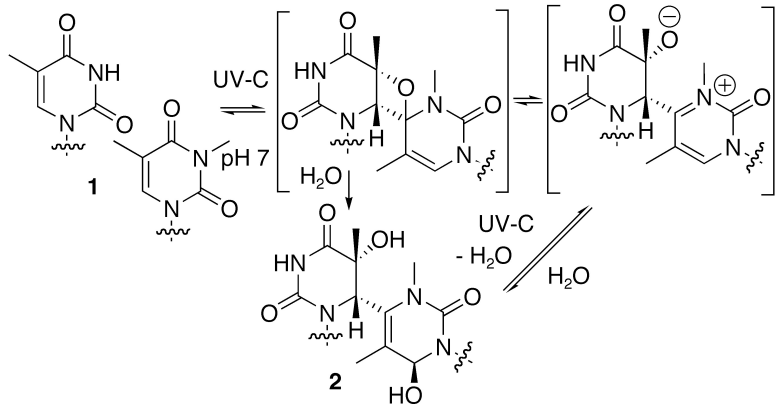

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Evidence That the (6−4) Photolyase Mechanism Can Proceed through an Oxetane Intermediate

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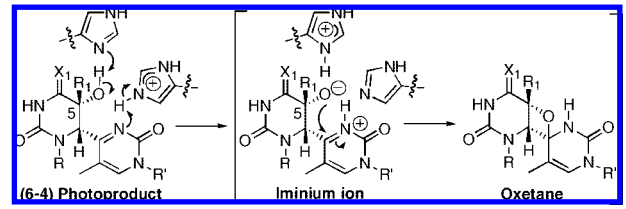
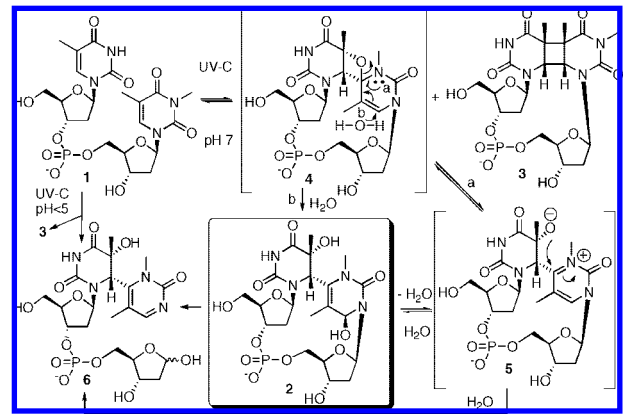
Pyrimidine (6−4) pyrimidone photoproducts ((6−4) PPs) are one of the main UV photoproducts formed in DNA under solar UV light.[1] This DNA photodamage, produced at two adjacent pyrimidines, is implicated in the etiology of skin cancer and photoageing.[2] Elucidation of the striking chemical structure of (6−4) PPs in 1967 simultaneously raised the problem of their formation mechanism. It is now accepted that (6−4) PPs result from the isomerization, through ring opening triggered by the deprotonation of N^3 of the 3′-pyrimidine, of an instable four-membered heterocycle intermediate initially produced by a Paternò-Büchi reaction between the C5−C6 double bond of a 5′-pyrimidine and the C4-heteroatom of a 3′-pyrimidine.[3] Whereas pieces of evidence for an unstable oxetane intermediate were reported in 1969,[4] the only experimental proof supporting this intricate mechanism was brought in the 1990s through the characterization of stable thietanes using the photochemistry of 4-thiothymine derivatives.[5] Recently, the discovery of a photolyase specific for the repair of (6−4) PPs has renewed the interest in the oxetane which is also supposed to be the key intermediate in the repair mechanism.[6] Oxetane formation from (6−4) PP is believed to be an acido/basic catalytic process triggered by the protonation of the N^3 atom of the pyrimidone moiety and deprotonation of the 5-OH of the 5-hydroxy-5,6-dihydrothymine residue by two histidine residues of the (6−4) photolyase (Scheme 1).[6d,e]

Surprisingly, despite the considerable interest currently devoted to the formation and repair mechanism of (6−4) PPs, neither successful experimental attempts at tackling the oxetane intermediate reactivity nor at synthesizing oxetane derivatives close to the biologically relevant one have ever been reported.[7] Herein, we provide new insights on formation of the putative oxetane and provide experimental evidence that complement the current model for the repair mechanism of (6−4) PPs by (6−4) photolyase. Guided by our photochemical studies in the 4-thiothymine series,[5b,8] we decided to investigate the photoreactivity of Tpm^{3}T (**1**)[9] at 254 nm anticipating that substitution of the N^3 atom of the 3′-end thymine residue should either stop the photochemical reaction at the oxetane level (**4**) (Scheme 2) or lead to the N^3-methyl iminium derivative (**5**) (Scheme 2, path a), a close analogue of the proposed iminium intermediate in the repair process (Scheme 1). UV−C irradiation of **1** in aqueous solution (pH 7, 10 mM Na phosphate) gave rise to **2** and **3** (Supporting Information Figures S4 and S9) isolated after HPLC in 15% and 22% yield, respectively, together with 57% yield of recovered **1**. Photoproduct **3** was identified as the cis-syn N^3-methyl cyclobutane photoproduct from its UV, mass

Scheme 1



Scheme 2



(HRMS (ESI, $(M + Na)^+$): calcd for $C_{21}H_{29}N_4O_{12}PNa$, 583.1417; found, 583.1423), and NMR data (S15−17), and comparison with literature.[10] For photoproduct **2**, absence of UV absorption maximum above 240 nm signed the loss of the two thymine chromophores. NMR data of the Tp- base moiety of **2** (δ H6 4.90, δ C6 58.7; δ_H CH$_3$ 1.66, δ_C CH$_3$ 28.4; δ C5 71.0)[11] were very close to those of the Tp- part of the (6−4) or Dewar of TpT.[10,12] However, NMR data of the -pm^{3}T base moiety of **2**[11] particularly δ C6 77.6 and C4 131.4 were not consistent with the oxetane structure **4**. Interestingly, δ C6 and δ C4 were in accordance with the presence of an hydroxyl group at the C6 position and a C4−C5 insaturation (δ C5 123.5 and δ C4 131.4). Therefore, we identified **2** as the N^3-methyl-C6 hydrate analogue of the (6−4) photoproduct of TpT. The ESI mass spectrum of **2** recorded in a methanol/H_2O mixture (negative mode, SI 25) afforded the expected ion at *m/z* 577 $(M - H)^-$ (calcd for $C_{21}H_{30}N_4O_{13}P$, 577.1547; found, 577.1560). The presence of an ion at *m/z* 559 indicated that this product was readily dehydrated (calcd for $C_{21}H_{28}N_4O_{12}P$, 559.1441; found, 559.1440). Fast exchange of the OH at C6 with a OCH_3, ascertained by the presence of an ion at *m/z* 591, (calcd for $C_{22}H_{32}N_4O_{13}P$, 591.1704; found, 591.1687) fully supported the expected chemical reactivity of **2**. MS/MS performed on the ions

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at *m/z* 591, 577, and 559 (SI 26) yielded a sole major ion at *m/z* 446 representing the loss of MeOH-H_2O-113, H_2O-113, and 113, respectively. Loss of a 113 neutral fragment is characteristic of the Tp- part of the (6−4) adduct of TpT.[13] NOEs between H6 and H3′ in each residue and between H6 Tp- and both CH_3 Tp- and C5-CH_3 -pm^{3}T indicated that the photochemical reaction arose between the two nucleoside residues in the anti glycosyl bond conformation and a C6 *S* configuration of the -pm^{3}T residue. Formation of **2** is fully consistent with the initial formation of an oxetane intermediate. From our observations, it is likely that oxetane **4** is so unstable that it leads to **2** either by addition of H_2O at C6 (path b, scheme 2) or via the 1,2-dihydro-2-oxopyrimidinium cation **5** (path a, Scheme 2), which is in equilibrium[14] with its pseudobase **2**. The diastereospecific formation of the C6 *S* isomer is in accordance with water addition on the less hindered side of the molecule.

Then, we anticipated that if a minute fraction of **2** was present as its 2-oxopyrimidinium cationic form (**5**), intramolecular Tp- C5 hydroxyl addition at position C4 of -pm^{3}T would lead to the oxetane that would reverse to **1** under 254 nm light (Scheme 2). Therefore, we exposed **2** to UV−C. To our delight, this led to its reversal to the parent dinucleotide **1** (S5).[15] Upon standing in H_2O at neutral pH, **2** gives rise to **6**. This derivative is directly obtained, together with **3**, when **1** is exposed to the 254 nm light in unbuffered water solution (pH 4.6) (S4, S18). In such conditions, **2** is not detected. The ^{1}H NMR spectrum of **6** displayed two singlets integrating for one proton each at δ 8.40 and 5.51 and two C5 methyl groups at δ 2.09 and 1.76 reminiscent of a (6−4) structure as also indicated by its UV absorption maximum at 321 nm. The molecular weight of **6** (HRMS (ESI, $(M - H)^-$): calcd for $C_{21}H_{30}N_4O_{13}P$, 577.1547; found, 577.1532) was 18 amu higher than that of **1**. Whereas the NMR data of the Tp-moiety of **6** were close to the corresponding part of **2**, hydrolysis of the 3′-end glycosyl bond of **6** was ascertained by the presence of split groups of signals for this sugar residue in ^{1}H and ^{13}C NMR spectra together with the typical chemical shift of its C1′ (δ 99.5).[16] The HMQC spectrum of **6** displayed a correlation between the proton at δ 8.50 attributed to H6 of the -pm^{3}T part and its carbon at δ 170.8. This H6 -pm^{3}T proton coupled with two quaternary carbons at δ 116.0 and δ 155.2, attributed respectively to C5 and C4. The chemical shifts of the -pm^{3}T base moiety are very close to the corresponding ones of the known 5, N^3-dimethyl-2-pyrimidinone motif.[8b] Therefore, **6** derives from the hydrolysis of the 3′-end *N*-glycosyl bond of **2** and aromatization, through dehydration of the -pm^{3}T motif and/or the direct hydrolysis of the 3′-end *N*-glycosyl bond of **5** that is in equilibrium with **2** (Scheme 2).

In conclusion, our results are of utmost importance with regard to the repair mechanism of (6−4) PPs by (6−4) photolyase. Indeed, we provide the first experimental evidence showing that as suggested, an iminium ion is able to restore the oxetane intermediate. Our study strongly reinforces the proposed model for (6−4) photoenzymatic repair and highlights a possible, and yet unsuspected, reactivity for the iminium ion intermediate that can readily react with water addition at the C6 position or at position C1′ of the 3′-end sugar residue. Spontaneous 3′-end *N*-glycosyl bond rupture is another outcome that may compete with oxetane formation within cellular DNA. This scenario would generate an abasic site which cannot be repaired by the (6−4) photolyase.

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Supporting Information Available: Experimental conditions for UV irradiation and HPLC, chromatograms of the photolysis of **1**, and the 254 nm photoreversion of **2** and **3**. NMR spectra of **1**, **2**, **3**, **6**, of the 254 nm photolysis of **1**, ESI MS data for **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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