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Photochemical Reactions of Microcrystalline Thymidine

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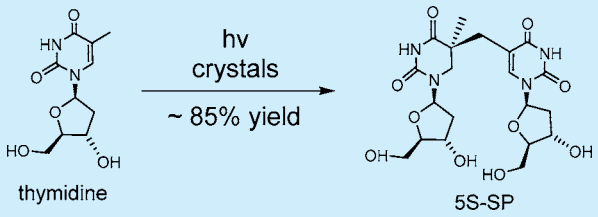
Supporting Information

ABSTRACT: Nucleoside/nucleotide/oligonucleotide photoreactions usually result in a number of products simultaneously due to a wide range of conformers existing at a given time. Such a complicated reaction pattern makes it difficult for one to focus on a single DNA photoproduct and elucidate the requirements for its formation. A rare example of thymidine photoreaction in microcrystals is reported, where 5-thyminyl-5,6-dihydrothymine, e.g., the spore photoproduct (SP), is produced as the dominant species in ∼85% yield. This unprecedented high yield clears the major obstacle for future SP photochemistry studies in detail.



DNA conformations are known to control the outcome of DNA photoreactions.[1−4] In a normal cell, the millions of nucleobases in the genome may adopt an equally large number of stacking conformations at a given time owing to the constant thermal motion. As a consequence, although cyclobutane pyrimidine dimers (CPDs) are usually the most abundant DNA photoproducts in a UV irradiated cell, their production is always accompanied by many other species. The lack of "clean" DNA photoproduct formation drastically hinders the understanding of the general DNA photochemistry.

To address this problem, unnatural nucleotides were adopted in in vitro DNA photochemical studies. For instance, using a dinucleotide TpT composed of locked nucleic acids in which the furanose moieties are locked at the C3′-endo conformation, an enhanced stacking interaction between the two thymine rings was achieved.[3] Such a favorable interaction eliminates the minor population of TpT conformers leading to the formation of pyrimidine (6−4) pyrimidone photoproduct (6−4PP),[3] the other common pyrimidine photolesion, resulting in CPD as the exclusive photoproduct upon UV irradiation.

Bacterial endospores represent an in vivo system where a certain type of DNA photoreaction is favored. Endospores are only ∼30% hydrated comparing with normal vegetative cells; such a low hydration level changes the genomic DNA to an A-conformation. The A-DNA is solidified by a group of DNA binding proteins named the small acid soluble proteins,[5−7] allowing the formation of 5-thyminyl-5,6-dihydrothymine, e.g., the spore photoproduct (SP), as the dominant photoproduct (>95%) upon UV irradiation.[8,9] A recent molecular simulation using the structure of a nucleoprotein formed between a small acid soluble protein and 10-mer oligo(dG)·oligo(dC) found that after replacing the sixth and seventh GC with AT pairs, the C5 of one T was only 3.4 Å away from the $-CH_3$ of another T.[7,10] This distance is shorter than the 3.9 Å between the two C5 positions, which is required to connect in CPD formation.[7] The corresponding moieties involved in the 6−4PP formation are even further. These results offer a rationale on how the DNA conformation promotes SP photochemistry and quenches other photoreactions.

SP can be formed in vitro via solid state (ice or dry film) DNA photoreactions; the numerous conformations adopted by thymine residues, however, determine that formation of SP is always accompanied by CPD and 6−4PP.[11−14] UV irradiation of thymidine at solid state produces dinucleoside SP.[13−16] Different from photoreactions in an oligonucleotide where the right-handed helix defines that only the 5*R*-SP can be generated,[8,17] the thymidine photoreaction results in a pair of SP diastereoisomers (Scheme 1).[15,16] As proved by Ames et al., these SP diastereoisomers are formed under the same H atom abstraction followed by radical recombination mechanism.[16]

Via slow evaporation of the thymidine methanol solution, Douki et al. obtained a thymidine thin film. UV irradiation of this film afforded the 5*R*- and 5*S*-SP in ∼1:20 ratio; few other thymidine photoproducts were produced. This result suggests that thymidine residues in the dry film stack into conformations favoring 5*S*-SP formation. However, the low yield (<1%) indicates that most of the thymidine residues in the dry film are nonreactive and release the excitation energy via thermal decay. This could be owing to the fact that only layers at the film surface are exposed to UV light while the bulk of the material is protected from reaction, although the possibility that the majority of thymidine residues adopt nonreactive conformations cannot be excluded. Crystals represent a special solid state, where molecules adopt a homogeneous structure within the whole

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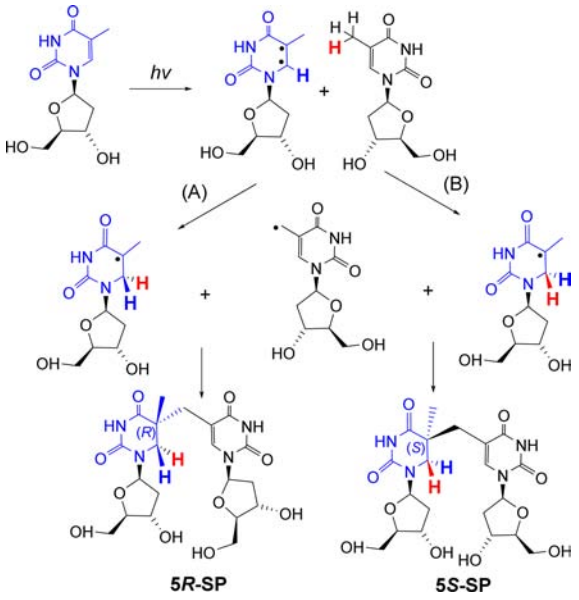
Scheme 1. Formation of Two SP Diastereoisomers upon Thymidine Dimerization



lattice. The high stereoselectivity and specificity of photoreactions in crystals are known for many years.[18,19] We thus wonder whether thymidine microcrystals can mimic the DNA environment in spores, leading to a clean SP formation under UV irradiation.

Following a literature protocol, we first prepared thymidine single crystals via slow evaporation of a thymidine aqueous solution.[20] Irradiation of the single crystals under unfiltered UV light centered at 254 nm for 1 h indeed results in one product at ≤0.1% yield; the only other species isolated via HPLC is the unreacted thymidine. This reaction thus represents a very rare example of "clean" nucleoside photoreaction. By comparing with the SP standards prepared by organic synthesis,[16] the product is confirmed as the 5*S*-SP. The low SP yield, however, indicates that the microcrystalline photoreaction may still suffer from the possible limitation of the dry film photoreaction that only the thymidine molecules near the surface react. To improve the reaction yield, we grinded the crystals into fine powder before UV light was applied. Even under a constant agitation, the yield of 5*S*-SP was still ∼1.0% after 24 h under 254 nm UV light (Figure 1A); a prolonged photoreaction led to little improvement.

To improve the yield of the crystalline photoreaction, Veerman et al. suspended the microcrystals of dicumyl ketone in water and irradiated the suspension under constant stirring.[21] A nearly stoichiometric conversion of reactant to product was obtained after 24 h of UV irradiation.[21] We therefore decided to adopt a similar strategy for our thymidine photoreaction. As thymidine is soluble in water, but not in many organic solvents, we mixed 2 mg of thymidine microcrystal powder with 2 mL of hexane and irradiated the suspension under 254 nm UV light with vigorous stirring in a cylinder quartz UV cuvette. To our delight, the yield of 5*S*-SP was improved drastically to 9.3% after a 2.5 h reaction.[22]

To reveal whether the solvents used alter the microcrystalline state and subsequently change the photoreaction yield, we surveyed a number of organic solvents, including diethyl ether, methyl *t*-butyl ether (MTBE), dicholomethane, dichloroethane, ethyl acetate, acetone, methanol, ethanol , etc., for the SP

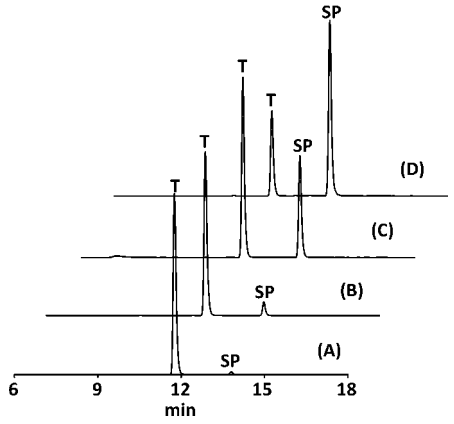


Figure 1. HPLC chromatograms for thymidine crystalline photoreactions under unfiltered UV light peaked at 302 nm; the reactions were monitored at 260 nm on HPLC. (A) In crystalline powder for 24 h and (B,C,D) in MTBE suspension for 12, 20, and 32 h, respectively. The 5*S*-SP was isolated in ∼85% yield after a 32 h irradiation. At 260 nm, the extinction coefficients of SP and thymidine are nearly identical.[12]

photoreaction.[22] MTBE was found to be the best solvent, resulting in 5*S*-SP at 30% yield after a 2.5 h reaction under 254 nm UV irradiation. We therefore chose MTBE for further thymidine photoreactivity studies.

Irradiation of 2 mg of thymidine microcrystals suspended in 2 mL of MTBE results in a linear formation of 5*S*-SP in the first several hours of the reaction (Figure 2). The reaction under

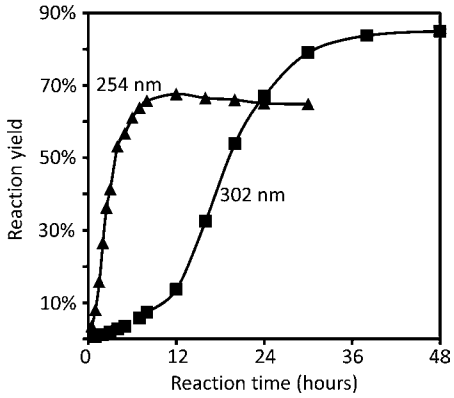


Figure 2. 5*S*-SP formation as a function of irradiation time for 2 mg of microcrystalline thymidine suspended in 2 mL of MTBE. A yield of 68% was achieved after a 12 h irradiation under unfiltered UV light peaked at 254 nm; while ∼85% yield can be obtained after 48 h under unfiltered UV light peaked at 302 nm. The "S" shape sigmoidal curve for both reactions suggests the presence of an "initiation" phase. A subtle conformational change in the thymidine crystal lattice induced by the formed SP molecules is indicated, which consequently makes the SP formation more favorable. The yield decrease after prolonged 254 nm UV irradiation is ascribed to possible SP decomposition.

unfiltered UV light peaked at 302 nm is ∼6-fold slower than that under the 254 nm UV light. Both reactions were very clean in the first 12 h; little other products were observed in the HPLC chromatograph (Figure 1B). The reaction under 254 nm UV light plateaued in 12 h with the yield of SP reaching 68 ± 3%. After 24 h, the yield decreased to ∼63% with subsequent formation of a number of new peaks in the HPLC chromatograph. We tentatively ascribe this observation to irradiation-induced SP decomposition processes. In contrast, although the

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reaction under 302 nm UV light was slower, the overall yield of SP is higher. An 84 ± 2% yield was obtained after a 32 h reaction. It is worth pointing out that both yields represent a drastic improvement comparing with SP formation in other *in vitro* systems, where the maximum yield observed was ~1%.[16,23]

The clean SP formation likely results from the homogeneous stacking conformation in the thymidine crystal, which quenches other side reactions. To reveal the thymidine conformation supporting 5*S*-SP formation, we reanalyzed the thymidine structure solved by Young et al.[20] The analysis reveals that the thymidine residues stack on each other to form different layers (Figure 3A). These layers are not perpendicular to the vector

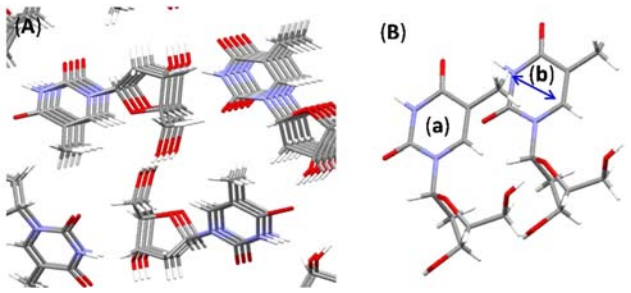

Figure 3. (A) Molecular packing in the thymidine single crystal. The distance between two thymidine rings is ~3.1 Å, close to the 3.36 Å average rise found in the SASP-oligo(dG)·oligo(dC) nucleoprotein.[7] (B) The shortest distance between an H atom in $-CH_3$ and the C6 of another thymine is ~3.2 Å (blue arrow), which is close to the 3.4 Å found in the molecular simulation[7] and supports the key H-abstraction step to initiate SP formation.[16,23]

defined by the centers of stacked thymine rings; instead they exhibit a dihedral angle of ~62°. Such a packing mode places the methyl group of one thymine (**a**, Figure 3B) right above the other thymine ring (**b**, Figure 3B); the shortest distance between an H atom of the $-CH_3$ moiety and the C6 of the adjacent thymine is only 3.178 Å. As indicated by the SP mechanism (Scheme 1),[16,23] the C6 radical formed after photoexcitation of the C5═C6 bond abstracts an H atom from the methyl moiety. Such an abstraction is highly feasible in the thymidine crystal as shown by the short distance revealed.

This packing mode also suggests that only the excited C5═C6 bond in ring **b** is positioned to abstract the H atom from the $-CH_3$ attached to ring **a** (Figure 3B); the reverse direction is prohibited. Therefore, only the 5*S*-SP can be formed in the thymidine crystalline photoreaction. Moreover, the C5═C6 bonds on adjacent thymidines are found to be 4.86 Å away from each other. Restricted by the crystal lattice, it is very difficult for them to move closer to enable the CPD formation. The C5═C6 bond is even further from the C4═O moiety of the adjacent thymidine (5.1−5.2 Å); the 6−4PP formation should also be inhibited. Consequently, the thymidine photoreaction in microcrystals is very clean; 5*S*-SP is produced as the dominant photoproduct.[22]

Although the thymidine structure provides key insights for the SP photochemistry, it may not represent the optimal reactive conformation. The SP formation exhibits an "S" shape sigmoidal curve (Figure 2) for both 254 and 302 nm reactions, contrasting to the hyperbolic curve found in the dicumyl ketone photoreaction.[21] The sigmoidal curve suggests a "positive cooperativity" among thymidine molecules in the crystal. Positive cooperativity is observed in multisubunit enzyme reactions, with the cooperative binding/releasing of dioxygen in hemoglobin the best known example.[24] Hemoglobin is a tetrameric enzyme where O_2 binding/releasing from the first subunit triggers protein conformational changes to activate other subunits, making the subsequent O_2 binding/releasing much easier. In a solid state reaction, an "S" shape curve may also be observed. Such a curve implies the presence of a "nucleation" phase, where reaction "hot spots" are generated to extend the interface between the reacted and unreacted regions in the crystalline lattice.[25] When enough "hot spots" are produced, the reaction enters the "growth" phase until most material is consumed. The reaction then slows down to enter the "deceleration" phase.[25]

The SP formation is triggered by photons, and the reaction is expected to be initiated evenly throughout the crystal lattice. The presence of the "nucleation" phase, however, indicates that although the stacking structure showing in Figure 3 supports SP photochemistry, it may not represent the optimal reactive conformation. The formation of SP may slightly alter the conformation of neighboring thymidine molecules, creating "hot spots" in the crystal lattice to accelerate reactions. Such an assumption is further supported by an incubation-followed-by-UV-irradiation experiment. Under the 302 nm UV light, the "growth" phase starts at ~12 h into the reaction (Figure 2). We thus dissolved the thymidine crystalline powder in MTBE and stirred the suspension for 14 h before UV light was applied. Analysis of SP formation revealed an identical reaction curve as that without such a pretreatment. Therefore, conformational changes due to a dissolution−crystallization process in MTBE can be ruled out. A subtle thymidine conformational change induced by SP formation, similar to the positive cooperativity in enzyme reactions, is likely responsible for the observed SP formation kinetics.

Such a positive cooperativity also offers an explanation to the different SP yields under 254 and 302 nm UV light. Although the 302 nm photoreaction is relatively slow, it also results in a slow crystal lattice change after SP formation, allowing more thymidine residues to adopt the reactive conformation and subsequently leading to a higher SP yield. In contrast, the faster SP formation under 254 nm UV light rapidly collapses the lattice, leaving more thymidine molecules at nonreactive conformations.

Different from the photodecarbonylation reaction of dicumyl ketone where nearly 100% conversion was observed,[21] the yield observed for our SP formation under 302 nm UV light is ~85%. We tentatively ascribe this yield difference to the different reaction pattern between these two photochemical systems. The dicumyl ketone photoreaction is an intramolecular reaction, which in theory can reach completion if being irradiated long enough. In contrast, the 5*S*-SP formation is an intermolecular reaction. The thymidine residues likely take a random order to react, and the reaction yield relies on the dimerization order. If every adjacent thymidine pair takes turns to dimerize, a 100% conversion to SP is expected. In contrast, if dimerization occurs at the first two of every three thymidine residues, the third thymidine will be left between two formed SPs and remain intact, resulting in the lowest possible yield at 67%. The actual yield of SP should fall in between the maximum and minimum yields, with 83%, the average, the most probable choice. Therefore, the ~85% SP formation under the 302 nm UV irradiation represents the optimal yield in the thymidine crystalline photoreaction. To our knowledge, such a high yield is unprecedented in nucleoside photochemistry.

In summary, we report the first microcrystalline nucleoside photoreaction using natural thymidine residues, which affords a clean formation of 5*S*-SP. Key information is deduced from the

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thymidine single crystal structure to explain the observed SP formation; analysis of the unique "S" shape reaction curve allows us to conclude that subtle conformational changes may occur to facilitate SP photochemistry. The ~85% yield in 302 nm reaction is unprecedented in DNA photochemical studies. This clean and high-yield SP formation opens the door for future SP photochemistry elucidation in detail. Further SP mechanistic investigations using thymidine microcrystals are currently underway.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis and characterization of 5*S*-SP product. 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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