



Unusually Large Deuterium Discrimination during Spore Photoproduct Formation

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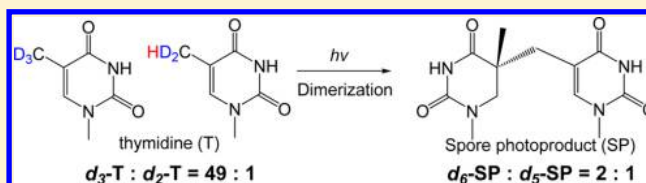
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S Supporting Information

ABSTRACT: The deuterium-labeling strategy has been widely used and proved highly effective in mechanistic investigation of chemical and biochemical reactions. However, it is often hampered by the incomplete label transfer, which subsequently obscures the mechanistic conclusion. During the study of photoinduced generation of 5-thymine-5,6-dihydrothymine, which is commonly called the spore photoproduct (SP), the Cadet laboratory found an incomplete (~67%) deuterium transfer in SP formation, which contrasts to the exclusive transfer observed by the Li laboratory. Here, we investigated this discrepancy by re-examining the SP formation using *d*₃-thymidine. We spiked the *d*₃-thymidine with varying amounts of unlabeled thymidine before the SP photochemistry is performed. Strikingly, our data show that the reaction is highly sensitive to the trace protiated thymidine in the starting material. As many as 17-fold enrichment is detected in the formed SP, which may explain the previously observed one-third protium incorporation. Although commercially available deuterated reagents are generally satisfactory as mechanistic probes, our results argue that attention is still needed to the possible interference from the trace protiated impurity, especially when the reaction yield is low and large isotopic discrimination is involved.

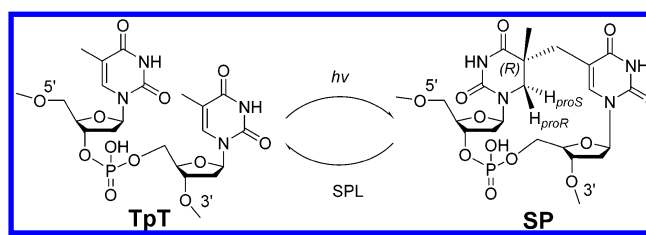


INTRODUCTION

The deuterium-labeling strategy is widely used in chemistry and biochemistry to probe the reaction mechanism, especially when several mechanistic routes are possible¹ or when the reaction is so sophisticated that no obvious reaction route can be deduced via simple structural analysis.² It is also very useful in studying metabolic pathways in living cells/microorganisms by feeding them with labeled substrates and following the label transfer during metabolic activities.³ Despite its wide usage, one of the potential problems in isotopic labeling experiments is the low yield. Incomplete deuterium transfer is often encountered with its molecular basis unknown. Although such observations can still provide useful mechanistic insight, the conclusion is far from being unambiguous.

Deuterium-labeling strategy was adopted in the mechanistic elucidation of the formation of a special thymine dimer, 5-thymine-5,6-dihydrothymine (Scheme 1), which is commonly called the spore photoproduct (SP) as it is the major DNA damage product found in UV-irradiated bacterial endospores.⁴ As much as 8% of the total genomic thymine residues can be converted to SPs,⁵ which is repaired by spore photoproduct lyase (SPL) during spore germination, ensuring their normal life cycle is not disrupted.^{4b,d,e,6} These SPs, if unrepaired, prove

Scheme 1



lethal to the germinated spores.⁷ Besides endospores, SP can be formed in UV-irradiated single-stranded and double-stranded oligonucleotides either in dry film or in ice,⁸ although its yield is very low (<1%).^{4c}

SP contains a chiral center at the C5 position of the 5'-T. In UV-irradiated oligomeric DNA, only one SP species, where the methyl group at the 3'-T is added to the C5 position of the 5'-T, with the newly formed C5 chiral center adopting an *R* configuration, has been isolated (Scheme 1).^{4b} In contrast, the lack of the phosphodiester linkage in thymidine photoreaction

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determines that either thymidine can undergo the addition reaction. Consequently, two SP stereoisomers adopting a *SR* and a *SS* configuration, respectively, are produced.⁹ To understand the mechanism of SP photochemistry, the Cadet group employed *d*₃-thymidine containing a deuterated methyl group for photoreaction and found that ~67% of the dinucleoside SP generated via UV irradiation in ice possessed a deuterium at the C6 carbon.^{4a,b} Although this result indicates an H atom transfer mechanism, the incomplete transfer observed hinders the determination of the origin of the two C6 protons on SP. The Li group recently examined the SP photochemistry using deuterium-labeled dinucleotide TpT in a dry film reaction and found an H atom on the methyl group of the 3'-T exclusively migrates to the 6proS position of the formed SP (Scheme 1, judged by signal integration in ¹H NMR spectra).^{4c}

Given that the SP photochemistry likely proceeds under a single mechanism, we are puzzled how the same labeling strategy results in different outcomes. We therefore investigated this discrepancy by re-examining the *d*₃-thymidine photoreaction to reveal what caused the incomplete deuterium transfer. We spiked the *d*₃-thymidine with varying amounts of unlabeled thymidine before the SP photochemistry is performed. Our results indicate that trideuterated thymidine is strongly discriminated in SP photochemistry. The trace protiated thymidine impurity is thus enriched into the SP product, which subsequently obscures the mechanistic conclusion. As deuterium labeling is such a common strategy used in mechanistic studies, our report here provides a useful reference for other "failed" experiments and/or strategies to improve the outcome of labeling studies.

RESULTS

Formation of Dinucleoside SP. The right-handed DNA helical structure and the restriction by the phosphodiester linkage between two thymine residues determine that only the (*SR*)-SP isomer can be generated after UV irradiation in an oligonucleotide.^{4b,e,8b} In contrast, loss of the phosphodiester moiety results in a pair of SP steric diastereomers, with the C5 chiral center adopting an *R* and an *S* configuration respectively in the thymidine photoreaction. The two dinucleoside SP isomers can be readily separated by reverse-phase HPLC.⁹ Moreover, different solid state (ice vs dry film) may alter the stacking interaction between the two thymine rings, which subsequently alters the relative yields of the two SP diastereomers.⁹ As shown by the Cadet group, in ice or in the dry film prepared by lyophilization of the thymidine aqueous solution, thymidine dimerization leads to two SP isomers with roughly equal yields. In contrast, photoreaction in the dry film made by ethanol evaporation resulted in one diastereomer as the dominant product. The steric configuration of this isomer, however, was unclear.

Similar results were observed here. The dry-film used in our studies was prepared by methanol evaporation; UV irradiation of this film produced the two diastereomers in ~1:3 ratio (Figure 1), which is slightly lower than the ~1:10 ratio previously reported by the Cadet laboratory.⁹ To assign the steric configuration for these SP isomers, we prepared the two diastereomeric dinucleosides via organic synthesis following protocols reported previously^{4c,6g,10} and determined their structures via NMR spectroscopy in agreement with earlier assignment.^{6g} Surprisingly, our results show that both thymidine photoreactions produce the *S* isomer as the major

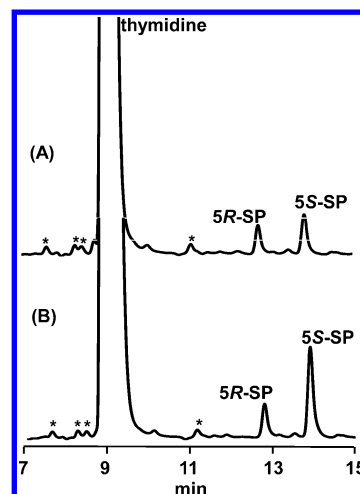


Figure 1. HPLC chromatograph of the two SP isomers generated from the thymidine photoreaction: (A) reaction in ice, (B) reaction in dry film prepared via methanol evaporation. The peaks denoted by * exhibit the same mass as the dinucleoside SP, suggesting that they are other thymine dimers such as cyclobutane pyrimidine dimers (CPDs) or pyrimidine (6–4) pyrimidone photoproducts (6–4PPs). These photoproducts are not the focus of this report and thus are not characterized further.

product, which is in sharp contrast to the fact that only *R* isomer was found in UV irradiated oligomeric DNA.^{4b,e,8b} Formation of the *R* diastereomer in oligonucleotide is ascribed to the natural tendency for DNA to adopt the right-handed helical structure. The dominance of the SP *S* isomer in the thymidine photoreaction here suggests that once the restriction asserted by the phosphodiester moiety is released, the favorable stacking interaction between the two thymine residues has changed, which leads to a conformation resulting in the *S* diastereomer. Moreover, although the SP isomers are produced as the major products in solid-phase photoreactions, their collective yields are still <1%, with the vast majority of thymidine residues (>98%) remaining unreacted. Such an observation can be ascribed to the fact that most of the thymidine residues adopt nonreactive conformations. After photoexcitation, these molecules cannot dimerize to SP; they are rapidly quenched via thermal relaxation instead.¹¹

No Drastic H Atom Exchange with Ice in SP Formation. After establishing the photoreaction conditions, we next examined the thymidine photoreaction in ice to reveal whether the previously observed one-third deuterium loss is due to an H atom/proton exchange with water. We used unlabeled thymidine to conduct the photoreaction in D₂O ice. If the deuterium loss is indeed due to the H atom exchange with water, we would expect obvious deuterium incorporation into the formed SPs. LC–MS analyses of the resulting SP molecules reveal that the intensity of the second isotopic peak accounts for 24.1 ± 0.4% and 24.9 ± 0.8% of the first one for the (*SR*) and (*SS*) isomers generated in the D₂O ice reaction at –78 °C, which are slightly above the theoretical value (23.8%) expected for a SP species with a natural isotope distribution suggested by the Agilent MassHunter software. Analysis of the “unreacted” thymidine residues indicates that the +1 signal in the mass spectrum has increased slightly; integration of the mass signals shows that 1 ± 0.1% of thymidine residues likely contain a deuterium. On the other hand, if starting with *d*₃-thymidine and irradiated the sample in H₂O ice for 2 h, ESI-MS

analysis of the unreacted thymidine suggests that the peak intensity of the -1 signal, corresponding to the product after the deuterium/protium exchange, increases by $1 \pm 0.1\%$ as well. The possible mechanism for such an H atom exchange reaction is unclear at this point. More importantly, although these observations suggest that minor H atom exchange with the environment may have occurred, the extent of exchange is still at the background level and is much lower than the one-third deuterium loss during SP formation observed in the previous study.^{4a,b} We therefore conclude that the H atom exchange from the environment has a negligible impact to the SP photoreaction discussed here.

Purity of the Synthesized d_3 -Thymidine. If the H atom/proton exchange reaction is not responsible for the 30–40% protium incorporation into the formed SP, the protium may come from the protiated thymidine impurity in the d_3 -thymidine used. We thus synthesized d_3 -thymidine by reacting the protected 2'-deoxyuridine with CD_3I , which has $\geq 99\%$ deuterium atom content as indicated by the vendor. The ESI-MS analysis of the resulting d_3 -thymidine suggests that $\sim 99.5\%$ of the thymidine molecules contain three deuterium atoms, $0.5 \pm 0.05\%$ contain two ($-CD_2H$), and $\sim 0.04\%$ contain no deuterium ($-CH_3$). The abundance of monodeuterated species ($-CDH_2$) is too low to be detected (Figure 2B).

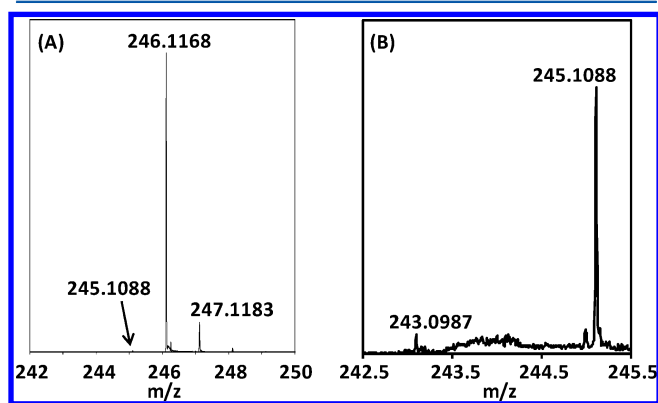
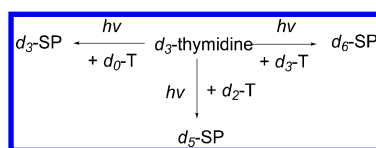


Figure 2. (A) LC–MS spectrum of the d_3 -thymidine under positive ion mode ($[M + H]^+$). The mass signal of 246.1168 corresponds to d_3 -thymidine and the signal of 245.1088 corresponds to d_2 -thymidine. (B) The zoom-in-view of the mass spectrum of the less deuterated thymidine species. The mass signal of 243.0987 corresponds to d_0 -thymidine. The abundance of the d_1 -thymidine species is too low to be estimated.

Three SP Isotopologues Are Expected. As d_3 -thymidine is the dominant species, the observed thymidine isotopologues likely dimerize with another molecule of d_3 -thymidine, producing the d_3 -, d_5 -, and d_6 -SP correspondingly (Scheme 2). The other SP isotopologues such as the d_0 - and the d_4 -SPs may not be detected due to the low abundances of d_0 - and d_2 -thymidine in the starting material. If the thymidine isotopologues are equally reactive, the isotopic distribution in

Scheme 2



the resulting SPs should resemble that of the d_3 -thymidine revealed by Figure 2. However, if some isotopologues are more reactive than the other, they will be selectively enriched in the formed SP products. This will result in an altered isotopic distribution pattern, which can be studied by mass spectrometry.

Enrichment of Protiated SP. We therefore dissolved the resulting d_3 -thymidine in H_2O , froze it at $-20^\circ C$, and exposed it to 254 nm UV light at $-78^\circ C$ for 30 min. The two SP isomers produced were analyzed by LC–MS using a Q-TOF mass spectrometer. As shown in Figure 2A, without zooming in the corresponding region, the d_2 -thymidine is buried underneath the baseline in the mass spectrum and barely observable. Surprisingly, its correspondent SP product, the (d_3 -SR)- and (d_5 -SS)-SPs, are clearly above the baseline. Comparing the signal intensities of d_2 -thymidine with d_3 -SPs in the mass spectra reveals that the d_2 -thymidine is enriched by 16.8 ± 1.5 fold for the (SR)-SP and 10.0 ± 0.7 fold for the (SS)-SP. When the reaction is conducted in dry film resulting from methanol evaporation at ambient temperature, protium enrichment is observed for about 7.5 fold for both the (SR)- and (SS)-SP diastereomers (Table 1). Examination of the formed d_3 -SPs

Table 1. Summary of d_0/d_2 -Thymidine Enrichment during SP Photoreaction in Ice at $-78^\circ C$ or in Dry Film at Ambient Temperature

	fold of enrichment for d_0 -thymidine		fold of enrichment for d_2 -thymidine	
	(SR)-SP	(SS)-SP	(SR)-SP	(SS)-SP
in ice ($-78^\circ C$)	16.8 ± 1.5	10.0 ± 0.7	14.9 ± 1.5	9.5 ± 1.0
in dry film ($25^\circ C$)	5.9 ± 0.9	6.5 ± 0.8	7.6 ± 0.9	7.3 ± 0.8

indicates that the unlabeled thymidine is drastically enriched as well (Figure 3). However, the extent of this enrichment is impossible to be deduced due to the low abundance of unlabeled thymidine in the starting material, making it difficult for us to accurately determine its amount in the d_3 -thymidine employed.

Thymidine Spike-in Experiments. To reveal how much the unlabeled thymidine is enriched during SP formation, we added unlabeled thymidine to the d_3 -thymidine used above until its final concentration reaches $0.2 \pm 0.01\%$, $0.5 \pm 0.03\%$, and $1 \pm 0.05\%$, respectively (Figure 4). As the concentration of d_2 -thymidine was determined to be $0.5 \pm 0.05\%$ as previously shown, we used it as the internal standard to quantify the amount of d_0 -thymidine added. Such a spike experiment yields three thymidine samples with the components accurately determined.

Subsequent photoreactions in ice at $-78^\circ C$ produced three major SP species, containing 3, 5, and 6 deuterium atoms, respectively. The ratios among these species can be accurately determined via mass signal integration (Figure 5). Again, d_2 -thymidine is enriched for 17-fold and 10-fold, respectively, for the (SR)- and (SS)-SP species, which are identical to those shown in the nonspiked experiments, confirming that the reaction pattern is unchanged after supplementation of the extra d_0 -thymidine. Judged by the mass signal intensity, it is clear that d_0 -thymidine and d_2 -thymidine are enriched similarly during the formation of SP (Table 1). In addition, a linear response between the amount of d_0 -thymidine included in the starting material and d_3 -SP generated are observed. The 17-fold

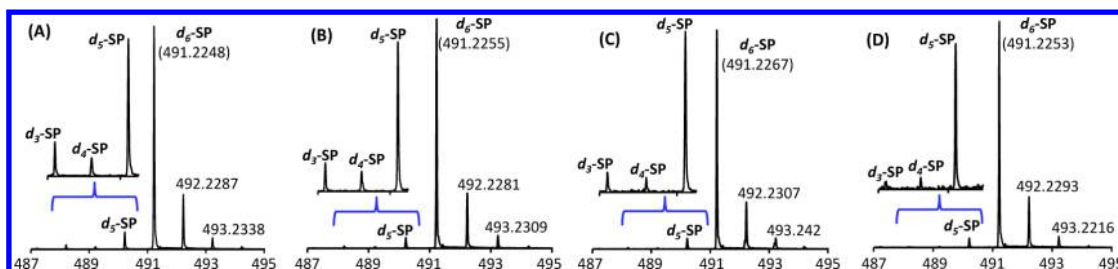


Figure 3. (A) LC–MS spectra of the SP isomers generated from the d_3 -thymidine photoreaction: (A) (SR)-SP generated in ice; (B) (SR)-SP generated in dry film; (C) (SS)-SP generated in ice; (D) (SS)-SP generated in dry film. All these spectra were recorded under positive-ion mode ($[M + H]^+$). The mass signals of 491.2, 490.2, and 488.2, correspond to the d_6 -, d_5 -, and d_3 -SP respectively.

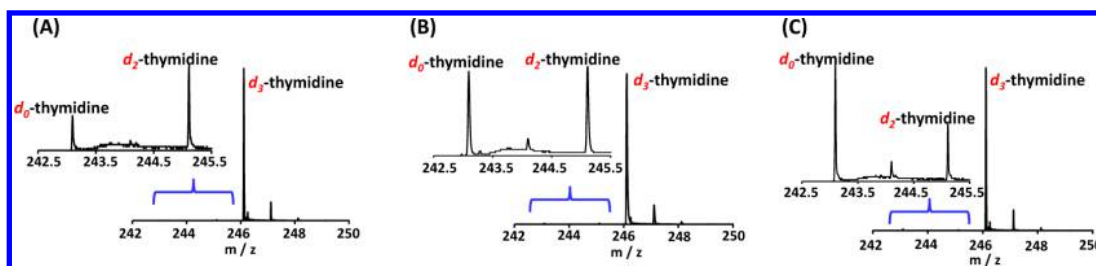


Figure 4. LC/MS spectra of the thymidine/ d_3 -thymidine mixture after being spiked by unlabeled thymidine. The unlabeled thymidine was added at a final concentration of 0.2% (A), 0.5% (B), and 1% (C). The d_2 -thymidine was determined previously to account for 0.5% of the samples and is used as the internal standard to quantify the unlabeled thymidine added.

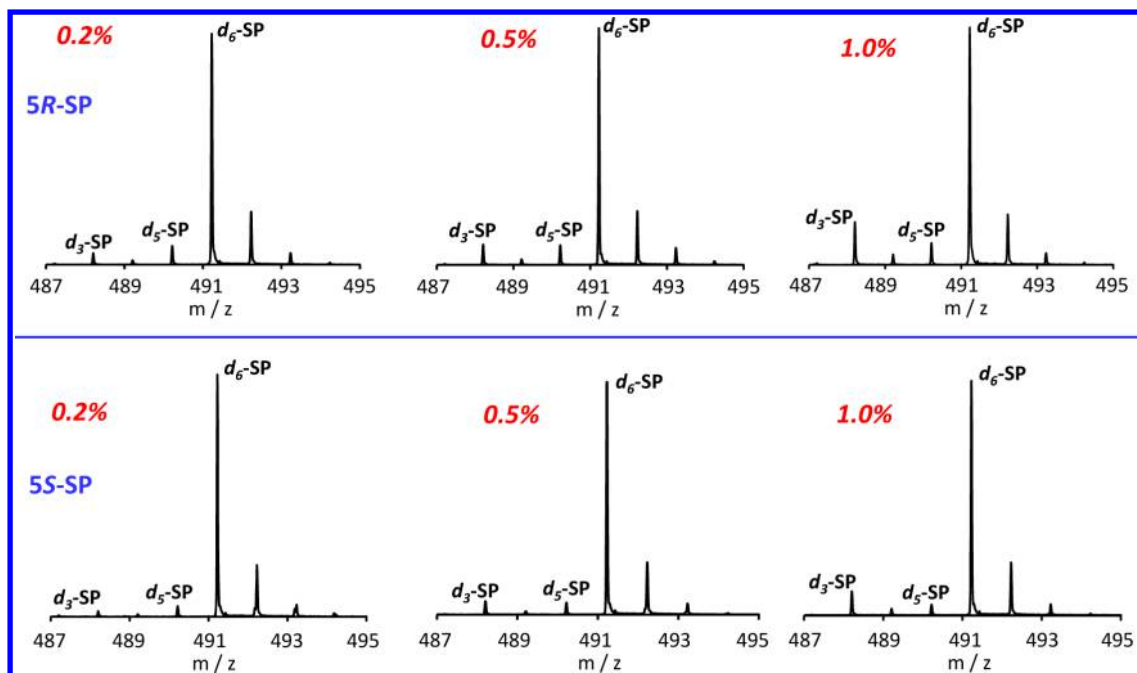


Figure 5. LC–MS spectra of the (SR)- and (SS)-SPs produced from the photoreaction in H_2O ice using the d_3 -thymidine (containing 0.5% d_2 -thymidine) spiked by various amounts of unlabeled thymidine. The fold of enrichment for protiated thymidine species during SP formation are listed in Table 1

protiated thymidine enrichment during (SR)-SP formation indicates that in order to obtain the one-third protiated SP observed in the previous experiment, only $\sim 2\%$ of protiated thymidine impurity is needed!

NMR Studies of SP Formed in the Dry Film Reaction.

The greater deuterium discrimination in the ice reaction determines that it is not the best means to study the H atom transfer process during SP formation using d_3 -thymidine residues. As shown in Table 1, less protium incorporation in the resulting SPs is achieved by the dry film photoreaction. We

therefore irradiated the thymidine in dry film to generate enough labeled SP products for NMR analyses, to reveal the origin of the H atoms on the C6 carbon of SP. The SPs produced by these dry film reactions still contain 6–7% protiated species; such a low content of impurities would not disturb our mechanistic investigation using NMR spectroscopy.

The C6 carbon in SP becomes pro-chiral. As a consequence, the two protons attached to that carbon are not equal, which result in two separated 1H NMR peaks. The coupling interaction between these two protons induces the 1H NMR

signal to split further, resulting in two doublets (Figure 6B & 6D). It is worth pointing out that as shown in Figure 6B,D, the

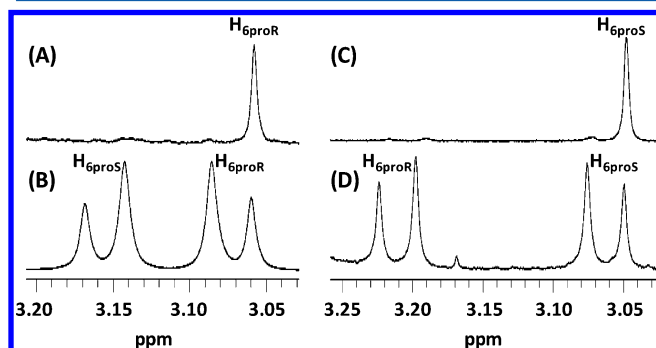


Figure 6. Zoom-in view of the ^1H NMR signals for the C6 protons in the (SR)- and (SS)-SPs generated by d_0 - or d_3 -thymidine photoreaction. All spectra were recorded in d_6 -DMSO: (A) (d_6 -5R)-SP; (B) (SR)-SP; (C) (d_6 -5S)-SP; (D) (SS)-SP. Please note that the chemical shifts for the $\text{H}_{6\text{proR}}$ and $\text{H}_{6\text{proS}}$ are reversed in the (SR)- and (SS)-SP species. Trace protiated SPs can be observed in the formed (d_6 -5R)- and (d_6 -5S)-SPs, agreeing with the mass spectrometric findings that the SPs produced by these dry film reactions contain 6–7% protiated molecules. Full NMR spectra for these SP species can be found in the Supporting Information.

chemical shifts for the $\text{H}_{6\text{proR}}$ and $\text{H}_{6\text{proS}}$ signals are reversed in the (SR)- and (SS)-SPs, which probably reflects the different

steric environment of these protons in the corresponding SP stereoisomers. The ^1H – ^1H coupling interaction is no longer present once one of the protons is replaced by a deuterium. As shown in Figure 6A, in the d_6 -5R SP produced, the $\text{H}_{6\text{proS}}$ signal disappears, and the remaining $\text{H}_{6\text{proR}}$ signal becomes a singlet. For the (d_6 -5S)-SP, the $\text{H}_{6\text{proR}}$ signal disappears, suggesting that the deuterium migrates to the 6proR position. These observations indicate that the formation of (SS)-SP mirrors that of the (SR)-isomer; the (SR)- and (SS)-SPs are produced under the same reaction mechanism.

To further verify the observed H atom transfer pattern in SP formation, we examined the proton correlations through space via ROESY spectroscopy for both (SR)- and (SS)-SP diastereomers. As shown in Figure 7A, in unlabeled (SR)-SP, the $\text{H}_{6\text{proR}}$ interacts with the $-\text{CH}_3$ moiety of the 3'-T, while $\text{H}_{6\text{proS}}$ associates with the two protons in the newly formed methylene bridge ($-\text{CH}_2-$). In the (d_6 -5R)-SP, the $\text{H}_{6\text{proS}}$ position is occupied by a deuterium. Consequently, the NMR signal due to the association between the $\text{H}_{6\text{proS}}$ and the methylene bridge disappears while the signals ascribed to $\text{H}_{6\text{proR}}$ remain (Figure 7C). Similarly, in the (d_6 -5S)-SP, the deuterium substitution at the $\text{H}_{6\text{proR}}$ position determines that the corresponding signals associated with that H atom disappear in the ROESY spectrum as well (Figure 7D). These observations agree with the results shown in Figure 6, establishing that an H atom from the thymine methyl moiety is transferred to the $\text{H}_{6\text{proS}}$ position during the (SR)-SP formation and to the $\text{H}_{6\text{proR}}$ position during (SS)-SP formation.

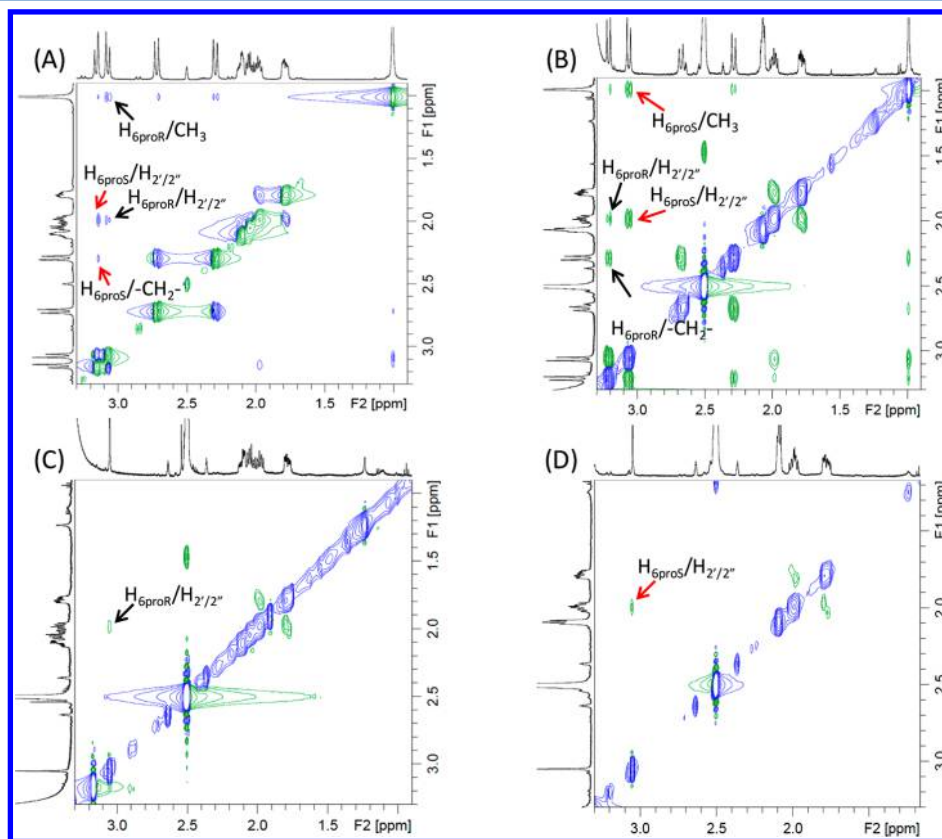
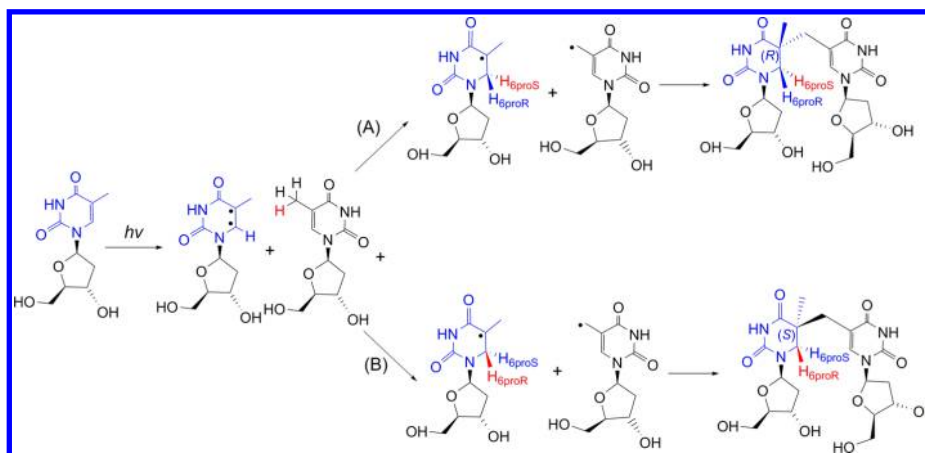


Figure 7. Zoom-in view of the ROESY spectra for the (SR)- and (SS)-SPs generated by d_0 - and d_3 -thymidine photoreactions, respectively. All spectra were recorded in d_6 -DMSO: (A) (SR)-SP; (B) (SS)-SP; (C) (d_6 -5R)-SP; (D) (d_6 -5S)-SP. The signals associated with $\text{H}_{6\text{proR}}$ are pointed by black arrows and the signals associated with $\text{H}_{6\text{proS}}$ are pointed by red arrows. The deuterium substitution at either the methyl or the C6 position causes the corresponding ROESY signal to disappear in the resulting d_6 -SP species. The full 2D-NMR spectra for these SPs can be found in the Supporting Information.

Scheme 3



DISCUSSION

Although SP was discovered nearly half a century ago,¹² the reaction mechanism for its formation has been debated for a long time.^{4b} The previous studies by the Cadet laboratory using d_3 -thymidine in ice found that one-third of the formed SP contained a protium at the C6 position. A later experiment by the Li laboratory using deuterium-labeled dinucleotide TpT in a dry film reaction found that a deuterium has been exclusively transferred to the C6 position.^{4c} The discrepancy between these two observations could be due to an H atom exchange with ice during the photoreaction; our data here, however, demonstrate that it is not likely the case. Instead, the protium from the trace amount of incompletely deuterated thymidine residues appears to be enriched drastically during SP photoreaction. Such enrichment is facilitated by the H_2O environment, which clearly favors the protium transfer comparing with that obtained from the dry film reaction.

The reduced extent of deuterium discrimination in the dry film environment allows a better examination of the SP formation. Starting with d_3 -thymidine, product analyses revealed that a deuterium is incorporated into the H_{6proS} position of (SR)-dinucleoside SP (Scheme 3). This result is consistent with our previous finding in the formation of the dinucleotide SP TpT, which only yields the (SR)-SP TpT.^{4c} Furthermore, a deuterium migrates from the methyl moiety to the H_{6proR} position of the (SS)-SP stereoisomer. These observations suggest the following. (i) Stereochemistry is strictly obeyed in the SP formation, probably due to the ultrafast nature of thymine dimerization reactions.^{11,13} The femtosecond dimerization rate and the even faster photoexcitation process ($\sim 10^{-15}$ s)¹⁴ determine that the reaction intermediates will not have time to change conformation. As a consequence, the reaction is highly stereoselective. (ii) H atom transfer reaction is the universal mechanism for SP formation. As revealed in Scheme 3, the lack of linkage between the thymidine residues implies that the two Ts can adopt two different stacking conformations, which determine not only the R and S configuration at the SP C5 chiral center but also the H atom configurations at the pro-chiral C6 center.

Although it is not surprising that the two SP stereoisomers form via the same H atom transfer mechanism, the unusually large deuterium discrimination during thymidine photoreaction is still very surprising. Under the ice reaction conditions adopted by the previous experiment, if the starting material d_3 -thymidine is 98% pure using some of the commercially available

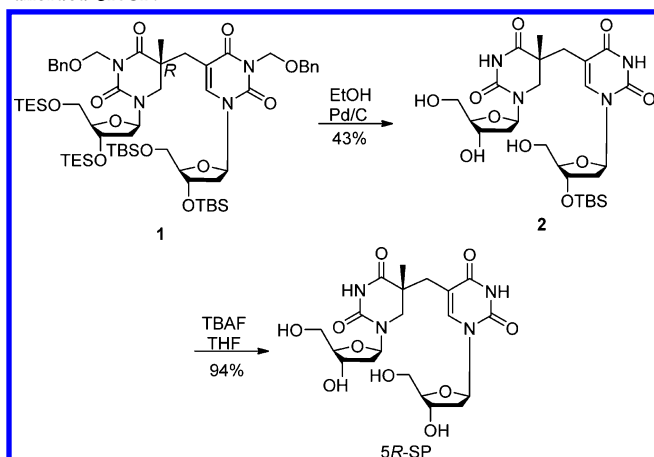
98% deuterated iodomethane, the 2% of protiated impurities can become 34% after the 17-fold enrichment in (SR)-SP formation. Such drastic enrichment could lead to ambiguous conclusions during mechanistic studies, especially when the product yield is low, as illustrated by the SP photoformation discussed here ($\leq 1\%$). Therefore, our result represents a very rare example to explain the “failed” labeling experiments, where the small amount of impurities may drastically interfere with the mechanistic investigation.

What is responsible for the dramatic deuterium discrimination during SP formation, especially for the photoreaction in ice? We tentatively ascribe this observation to an unexpected large kinetic isotope effect (KIE). The SP formation exhibits a kinetic isotope effect of 3.5, as revealed by our dinucleotide TpT photoreaction.^{4c} In the dinucleotide TpT, although the distance between the two thymine residues may be determined by the ring-stacking interaction, it is likely influenced by the phosphodiester linker as well. A piece of evidence supporting this assumption is the deuterium-transfer pattern in dry film reactions. In the thymidine photoreaction discussed in this report, the deuterium is discriminated by 6–7-fold. In contrast, judged by NMR spectroscopy, no detectable deuterium enrichment was observed in the dinucleotide TpT photoreaction.^{4c} In H_2O , the hydrophilic environment likely forces the two thymine residues, which have a hydrophobic aromatic ring to stack more tightly. As the consequence, the distance between these two thymine rings will probably be even smaller in frozen aqueous solution than that in the dry film. If the large deuterium discrimination observed in our current studies is indeed due to the primary kinetic isotope effect, its large scale suggests that H atom tunneling mechanism may be encountered. As the tunneling effect is affected by the distance between the two reacting moieties,¹⁵ the different KIEs observed here may be ascribed to the distance resulted from the different extents of stacking interaction. Such an explanation is also supported by the different KIEs observed in the formation of (SR)- and (SS)-SP diastereomers generated from the same photoreaction; the different yields of R and S stereoisomers from the SP formation in ice or in dry film indicate different stacking interactions between two thymine residues which favor the formation of two different isomers. Further theoretical or experimental studies are needed in the future to test the validity of this hypothesis.

EXPERIMENTAL SECTION

Chemicals. Unless otherwise stated, all solvents and chemicals used were of commercially available analytical grade and used without further purification. Purification of reaction products was carried out by flash chromatography using silica gel. The ^1H and ^{13}C NMR spectra were obtained on a 500 MHz NMR spectrometer. The photoreaction was carried out using a germicidal UV lamp (Dual-tube, 15 w, intensity: 1550 uw/cm^2) with samples $\sim 5 \text{ cm}$ from the lamp.^{4c} High-performance liquid chromatography (HPLC) was performed at room temperature with a UV/vis detector at 268 nm. A C18 column ($2.5 \mu\text{M}$, $4.6 \times 50 \text{ mm}$) was used for product analysis and another one ($2.5 \mu\text{M}$, $10 \times 50 \text{ mm}$) for product preparation on milligram scale. The regular mass spectrometry (MS) analysis was obtained with electrospray ionization (ESI). The high-resolution LC-MS analyses were conducted via a Q-TOF LC/MS coupled to a capillary chromatography system. The high-resolution mass data analysis and formula assignments were conducted using the MassHunter software.

Synthesis of Dinucleoside 5R-SP. The synthesis of compound 1 was achieved using published procedures (the 5R configuration has been confirmed by X-ray crystal diffraction analysis).¹⁶ Subsequent Pd/C-catalyzed hydrogenation and deprotection of the TBS group afforded 5R-SP.



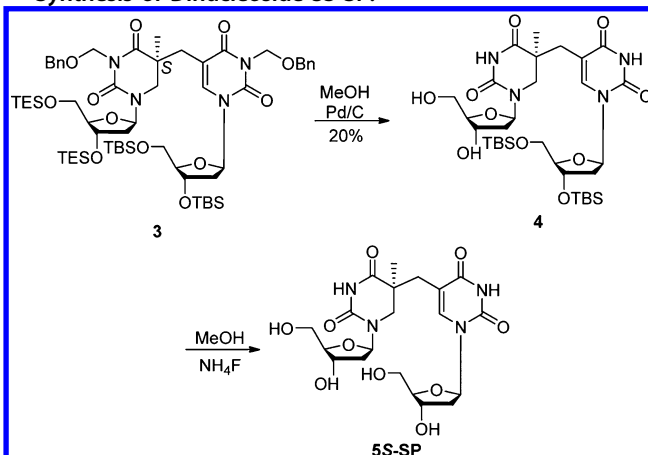
Synthesis of Compound 1. The synthesis of compound 1 was achieved using published procedures.^{4c} 5R-1: ^1H NMR (CDCl_3) δ 0.08 (s, 3H), 0.09 (s, 3H), 0.12 (s, 6H), 0.60 (q, $J = 7.9 \text{ Hz}$, 6H), 0.65 (q, $J = 8.0 \text{ Hz}$, 6H), 0.89 (s, 9H), 0.93 (s, 9H), 0.96 (t, $J = 8.0 \text{ Hz}$, 9H), 0.98 (t, $J = 7.9 \text{ Hz}$, 9H), 1.18 (s, 3H), 1.85–1.98 (m, 3H), 2.10–2.18 (m, 1H), 2.62 (d, $J = 14.1 \text{ Hz}$, 1H), 2.68 (d, $J = 14.1 \text{ Hz}$, 1H), 2.91 (d, $J = 13.0 \text{ Hz}$, 1H), 3.11 (d, $J = 13.0 \text{ Hz}$, 1H), 3.60 (dd, $J = 5.2$, 10.4 Hz , 1H), 3.68 (dd, $J = 3.3$, 10.7 Hz , 1H), 3.74 (dd, $J = 5.0$, 10.7 Hz , 1H), 3.77–3.83 (m, 2H), 3.86–3.92 (m, 1H), 4.29–4.33 (m, 1H), 4.39–4.44 (m, 1H), 4.55 (d, $J = 12.4 \text{ Hz}$, 1H), 4.61 (d, $J = 12.4 \text{ Hz}$, 1H), 4.66 (d, $J = 12.1 \text{ Hz}$, 1H), 4.69 (d, $J = 12.1 \text{ Hz}$, 1H), 5.32 (s, 2H), 5.47 (s, 2H), 6.30 (t, $J = 6.6 \text{ Hz}$, 1H), 6.33 (t, $J = 7.1 \text{ Hz}$, 1H), 7.20–7.27 (m, 6H), 7.27–7.32 (m, 2H), 7.32–7.37 (m, 2H), 7.49 (s, 1H); ^{13}C NMR (CDCl_3) δ -5.3, -5.2, -4.8, -4.6, 4.4, 4.8, 6.8, 6.9, 18.0, 18.5, 22.1, 25.8, 26.1, 32.4, 36.9, 40.2, 43.0, 44.2, 63.0, 63.1, 70.9, 71.9, 72.0, 72.2, 72.3, 84.4, 85.4, 86.4, 87.6, 108.6, 127.5, 127.6, 127.7, 128.3, 128.4, 138.1, 138.5, 139.3, 150.8, 152.4, 163.6, 173.7; ESI-MS (positive ion) calcd for $\text{C}_{60}\text{H}_{101}\text{N}_4\text{O}_{12}\text{Si}_4^+$ 1181.6 ($\text{M} + \text{H}^+$), found 1181.6.

Synthesis of Compound 2. A solution of 1 (500 mg, 0.42 mmol) and 10% Pd/C (150 mg) in EtOH (20 mL) was stirred under 1 atm hydrogen gas for 1 day. After filtration and concentration under vacuum, the residue was purified by flash chromatography (eluent: hexane/EtOAc/MeOH = 1/1/0.2) to afford compound 2 as a white oil (108 mg, 0.18 mmol, 43%); ESI-MS (positive ion) calcd for $\text{C}_{26}\text{H}_{43}\text{N}_4\text{O}_{10}\text{Si}^+$ 599.7 ($\text{M} + \text{H}^+$), found 599.7. ^1H NMR (d_4 -methanol) δ 0.12 (s, 6H), 0.91 (s, 9H), 1.18 (s, 3H), 1.97 (ddd, $J = 3.1$, 6.3, 13.6 Hz , 1H), 2.14–2.24 (m, 3H), 2.53 (d, $J = 14.1 \text{ Hz}$, 1H), 2.79 (d, $J = 14.1 \text{ Hz}$, 1H), 3.26 (d, $J = 13.1 \text{ Hz}$, 1H), 3.33 (d, $J = 13.1 \text{ Hz}$, 1H), 3.60 (dd, $J = 4.7$, 11.9 Hz , 1H), 3.64 (dd, $J = 4.2$, 11.9 Hz , 1H), 3.71 (dd, $J = 3.7$, 12.0 Hz , 1H), 3.74–3.79 (m, 2H), 3.89–3.93

(m, 1H), 4.25–4.29 (m, 1H), 4.49–4.53 (m, 1H), 6.24 (t, $J = 6.7 \text{ Hz}$, 1H), 6.25 (t, $J = 6.7 \text{ Hz}$, 1H), 7.86 (s, 1H); ^{13}C NMR (d_4 -methanol) δ -4.7, -4.5, 18.8, 21.3, 26.2, 32.3, 37.3, 41.8, 43.2, 47.2, 62.6, 63.4, 72.5, 73.6, 85.2, 86.5, 87.3, 89.4, 110.4, 141.4, 152.0, 154.4, 166.1, 176.5; HRMS (MH^+) calcd for $\text{C}_{26}\text{H}_{43}\text{N}_4\text{O}_{10}\text{Si}^+$ 599.2740 ($\text{M} + \text{H}^+$), found 599.2728.

Synthesis of 5R-SP. TBAF (1.0 M in THF, 0.50 mL) was added to a solution of compound 2 (100 mg, 0.17 mmol) in THF (10 mL) and stirred for overnight. After solvent evaporation under vacuum, the residue was then purified by reversed-phase HPLC in the gradient mode using water/ CH_3CN as solvents. The compound 5R-SP was isolated as a white solid (77 mg, 0.16 mmol, 94%); ^1H NMR ($\text{DMSO}-d_6$) δ 1.01 (s, 3H), 1.79 (ddd, $J = 2.8$, 6.1, 12.9 Hz , 1H), 1.93–2.15 (m, 3H), 2.29 (d, $J = 13.9 \text{ Hz}$, 1H), 2.72 (d, $J = 13.9 \text{ Hz}$, 1H), 3.07 (d, $J = 13.0 \text{ Hz}$, 1H), 3.16 (d, $J = 13.0 \text{ Hz}$, 1H), 3.33–3.48 (m, 2H), 3.51–3.63 (m, 3H), 3.75–3.81 (m, 1H), 4.06–4.14 (m, 1H), 4.21–4.28 (m, 1H), 4.72 (brs, 1H), 5.01 (brs, 1H), 5.12 (brs, 1H), 5.24 (brs, 1H), 6.11 (t, $J = 7.2 \text{ Hz}$, 1H), 6.16 (t, $J = 6.8 \text{ Hz}$, 1H), 7.69 (s, 1H), 10.12 (s, 1H), 11.22 (brs, 1H); ^{13}C NMR ($\text{DMSO}-d_6$) δ 20.2, 30.8, 35.8, 39.8, 41.1, 45.5, 61.5, 62.0, 70.7, 70.8, 83.0, 84.1, 85.9, 87.5, 108.7, 139.1, 150.4, 152.5, 163.8, 174.4; ESI-MS (positive ion) calcd for $\text{C}_{20}\text{H}_{29}\text{N}_4\text{O}_{10}^+$ 485.2 ($\text{M} + \text{H}^+$), found 485.2.

Synthesis of Dinucleoside 5S-SP.



Synthesis of Compound 3. The synthesis of compound 3 was achieved using published procedures.¹⁶ 5S-3: ^1H NMR (CDCl_3) δ 0.09 (s, 12H), 0.58–0.66 (m, 12H), 0.90 (s, 18H), 0.97 (t, $J = 7.9 \text{ Hz}$, 18H), 1.11 (s, 3H), 1.88–2.00 (m, 3H), 2.28 (dd, $J = 4.7$, 12.7 Hz , 1H), 2.43 (d, $J = 14.2 \text{ Hz}$, 1H), 2.78 (d, $J = 13.0 \text{ Hz}$, 1H), 2.79 (d, $J = 14.2 \text{ Hz}$, 1H), 3.18 (d, $J = 13.0 \text{ Hz}$, 1H), 3.59 (dd, $J = 4.9$, 10.4 Hz , 1H), 3.65–3.80 (m, 3H), 3.81–3.87 (m, 1H), 3.90–3.96 (m, 1H), 4.30–4.35 (m, 1H), 4.35–4.39 (m, 1H), 4.61 (s, 2H), 4.65 (s, 2H), 5.25 (d, $J = 9.9 \text{ Hz}$, 1H), 5.37 (d, $J = 9.9 \text{ Hz}$, 1H), 5.44 (s, 2H), 6.23 (t, $J = 6.7 \text{ Hz}$, 1H), 6.37 (t, $J = 7.0 \text{ Hz}$, 1H), 7.20–7.33 (m, 8H), 7.33–7.38 (m, 2H), 7.41 (s, 1H); ^{13}C NMR (CDCl_3) δ -5.4, -5.2, -4.7, -4.6, 4.4, 4.8, 6.8, 6.9, 18.0, 18.4, 20.8, 25.8, 26.0, 32.5, 37.2, 40.8, 42.3, 44.7, 63.4, 63.5, 70.7, 71.0, 71.9, 72.2, 72.8, 72.9, 85.0, 86.3, 86.8, 88.1, 108.6, 127.4, 127.5, 127.6, 127.7, 128.3, 128.4, 138.0, 138.1, 138.9, 150.7, 152.5, 163.4, 173.5; ESI-MS (positive ion) calcd for $\text{C}_{60}\text{H}_{101}\text{N}_4\text{O}_{12}\text{Si}_4^+$ 1181.6 ($\text{M} + \text{H}^+$), found 1181.6.

Synthesis of Compound 4. A solution of 3 (200 mg, 0.17 mmol) and 10% Pd/C (20 mg) in MeOH (10 mL) at 40°C was stirred under 1 atm hydrogen gas for 2 days. After filtration and concentration under vacuum, the residue was purified by flash chromatography (eluent: hexane/EtOAc/MeOH = 1/1/0.2) to afford compound 4 as a colorless solid (10 mg, 0.014 mmol, 8%); ^1H NMR (CDCl_3) δ 0.07 (s, 3H), 0.08 (s, 3H), 0.11 (s, 3H), 0.13 (s, 3H), 0.89 (s, 9H), 0.91 (s, 9H), 1.24 (s, 3H), 1.99 (ddd, $J = 5.6$, 8.0, 13.5 Hz , 1H), 2.18–2.34 (m, 3H), 2.66 (d, $J = 14.1 \text{ Hz}$, 1H), 2.76 (d, $J = 14.1 \text{ Hz}$, 1H), 3.21 (d, $J = 13.1 \text{ Hz}$, 1H), 3.32 (d, $J = 13.1 \text{ Hz}$, 1H), 3.63–3.70 (m, 2H), 3.70–3.79 (m, 3H), 3.83 (dd, $J = 3.8$, 11.2 Hz , 1H), 3.98–4.01 (m, 1H), 4.37–4.40 (m, 1H), 4.49–4.53 (m, 1H), 6.21 (dd, $J = 6.0$, 8.4 Hz , 1H), 6.22 (t, $J = 6.7 \text{ Hz}$, 1H), 7.66 (s, 1H), 7.94 (s, 1H), 10.41 (s, 1H); ^{13}C

NMR (CDCl₃) δ -5.3, -4.8, -4.7, 14.1, 18.0, 18.4, 22.2, 25.8, 25.95, 26.00, 29.7, 31.2, 36.9, 41.3, 46.5, 61.6, 63.5, 70.3, 73.0, 84.2, 85.3, 86.6, 88.5, 109.1, 140.2, 150.2, 152.8, 165.3, 173.7; HRMS (MH⁺) calcd for C₃₂H₅₇N₄O₁₀Si₂⁺ 713.3613 (M + H⁺), found 713.3620.

Synthesis of 5S-SP. A solution of compound 4 (10 mg, 0.014 mmol) and NH₄F (6 mg, 0.14 mmol) in MeOH (10 mL) was stirred overnight, dried under reduced pressure, and purified by reversed-phase HPLC in the gradient mode using water/CH₃CN. 5S-SP was isolated as a colorless solid (6 mg, 0.012 mmol, 90%): ¹H NMR (DMSO-*d*₆) δ 0.99 (s, 3H), 1.17 (ddd, *J* = 2.8, 6.1, 13.1 Hz, 1H), 1.99 (ddd, *J* = 6.2, 8.2, 13.5 Hz, 1H), 2.06–2.11 (m, 3H), 2.26 (d, *J* = 13.9 Hz, 1H), 2.72 (d, *J* = 13.9 Hz, 1H), 3.07 (d, *J* = 13.0 Hz, 1H), 3.21 (d, *J* = 13.0 Hz, 1H), 3.38–3.48 (m, 2H), 3.51–3.63 (m, 3H), 3.77 (dd, *J* = 4.0, 7.0 Hz, 1H), 4.07–4.14 (m, 1H), 4.21–4.28 (m, 1H), 4.72 (brs, 1H), 5.01 (brs, 1H), 5.12 (brs, 1H), 5.24 (brs, 1H), 6.12 (dd, *J* = 6.4, 8.0 Hz, 1H), 6.16 (t, *J* = 7.2 Hz, 1H), 7.60 (s, 1H), 10.12 (s, 1H), 11.22 (brs, 1H); ¹³C NMR (DMSO-*d*₆) δ 19.4, 30.7, 31.0, 35.8, 41.1, 61.5, 62.0, 63.1, 70.6, 70.8, 82.9, 84.0, 86.0, 87.3, 99.5, 108.6, 138.8, 152.3, 174.1; ESI-MS (positive ion) calcd for C₂₀H₂₉N₄O₁₀⁺ 485.2 (M + H⁺), found 485.2.

Preparation of d₃-Thymidine. Synthesis of d₃-thymidine was conducted as previously described using 2'-deoxyuridine and iodomethane-d₃ ($\geq 99\%$).^{4c}

UV Irradiation of Thymidine. The photochemical preparation of dinucleosides 5R- and 5S-SPs were conducted under modified literature experimental procedures.^{4c,6g,10} Briefly, 15 mL of a 1 mM thymidine solution in MeOH was transferred to a 15 × 20 cm glass plate. Methanol evaporation afforded a nice thin film. The film was then exposed for 30 min to UVC radiation (254 nm) light. In a separate set of experiments, thymidine was dissolved in 5 mL of dd H₂O or D₂O to a final concentration of 1 mM. The resulting solution was transferred to a 10 cm Petri dish, frozen in a -20 °C freezer, and irradiated on dry ice (-78 °C) by exposing to UVC light for 30 min. The formed SPs were then analyzed by HPLC-MS.

The products were analyzed by HPLC-MS under the positive-ion mode. For the HPLC part, the column was equilibrated in solvent A (water), and compounds were eluted with an ascending gradient (1–10%) of acetonitrile (solvent B) in 20 min at a flow rate of 1 mL/min. Under this gradient, thymidine was eluted at 9.2 min, (5R)-SP at 12.9 min, and (5S)-SP at 14.0 min. The identity of the products was further confirmed by coinjection of respective authentic samples prepared by organic synthesis.

To obtain enough samples for NMR analysis, the experiments were repeated 6 times and products separated by semipreparative HPLC under gradient mode using water and acetonitrile as elution solvents. The gradient changed from 2% to 10% B in 15 min at a flow rate of 4.73 mL/min. Under this gradient, thymidine was eluted at 3.0 min, (5R)-SP at 4.5 min, and (5S)-SP at 5.8 min.

■ ASSOCIATED CONTENT

■ Supporting Information

¹H and ¹³C NMR characterizations of the (5R)- and (5S)-SPs. 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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