

Photosensitized [2 + 2] cycloaddition of *N*-acetylated cytosine affords stereoselective formation of cyclobutane pyrimidine dimer

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ABSTRACT

Photocycloaddition between two adjacent bases in DNA produces a cyclobutane pyrimidine dimer (CPD), which is one of the major UV-induced DNA lesions, with either the *cis-syn* or *trans-syn* structure. In this study, we investigated the photosensitized intramolecular cycloaddition of partially-protected thymidylyl-(3'→5')-*N*⁴-acetyl-2'-deoxy-5-methylcytidine, to clarify the effect of the base modification on the cycloaddition reaction. The reaction resulted in the stereoselective formation of the *trans-syn* CPD, followed by hydrolysis of the acetyl amino group. The same result was obtained for the photocycloaddition of thymidylyl-(3'→5')-*N*⁴-acetyl-2'-deoxycytidine, whereas both the *cis-syn* and *trans-syn* CPDs were formed from thymidylyl-(3'→5')-thymidine. Kinetic analyses revealed that the activation energy of the acid-catalyzed hydrolysis is comparable to that reported for the thymine-cytosine CPD. These findings provided a new strategy for the synthesis of oligonucleotides containing the *trans-syn* CPD. Using the synthesized oligonucleotide, translesion synthesis by human DNA polymerase η was analyzed.

INTRODUCTION

In the field of organic synthesis, cycloaddition reactions are versatile and well-studied methods to form carbon–carbon covalent bonds. They include the [2 + 2] cycloaddition by light irradiation, the Diels–Alder reaction by

heating, and the 1,3-dipolar cycloaddition using metal catalysts. Among such reactions, the [2 + 2] cycloaddition, in which 4 π electrons of two double bonds participate in the formation of a four-membered ring, is widely used for the construction of the core four-membered ring in natural products and for the introduction of functional groups by rearrangement via the heterolytic opening of the strained four-membered ring (1–5).

DNA is an organic molecule that undergoes the [2 + 2] cycloaddition reaction as well as other chemical reactions. The major reaction occurs between the C5–C6 double bonds of adjacent pyrimidine bases when they are exposed to environmental ultraviolet light, which results in the formation of cyclobutane pyrimidine dimers (CPDs), a major type of UV-induced lesions. The structure of the CPD can be categorized into four types, namely *cis-syn*, *trans-syn*, *cis-anti* and *trans-anti*, depending on the spatial alignment of the two bases (Figure 1A). Among these four structures, the *cis-syn* CPD is predominantly formed in double-stranded DNA, due to geometric constraints (6,7). The *trans-syn* CPD, which is formed by UVB and UVC at partially distorted regions within DNA (8–11), is the second major stereoisomer of this photo-product. This type of CPD is reportedly recognized by proteins (12–14). The *trans-syn* CPD is a substrate for DNA repair enzymes, such as DNA photolyase (12) and T4 endonuclease V (13), although the repair efficiency was much lower than that for the *cis-syn* CPD. UvrA and XPE, which are the prokaryotic and human proteins involved in nucleotide excision repair, respectively, bound to this CPD with an affinity comparable to that for other UV lesions (14). Other types of CPD structures are reportedly formed between non-adjacent bases (15,16). UV irradiation of a single-stranded oligonucleotide in an acidic solution and the telomeric G-quadruplex structure

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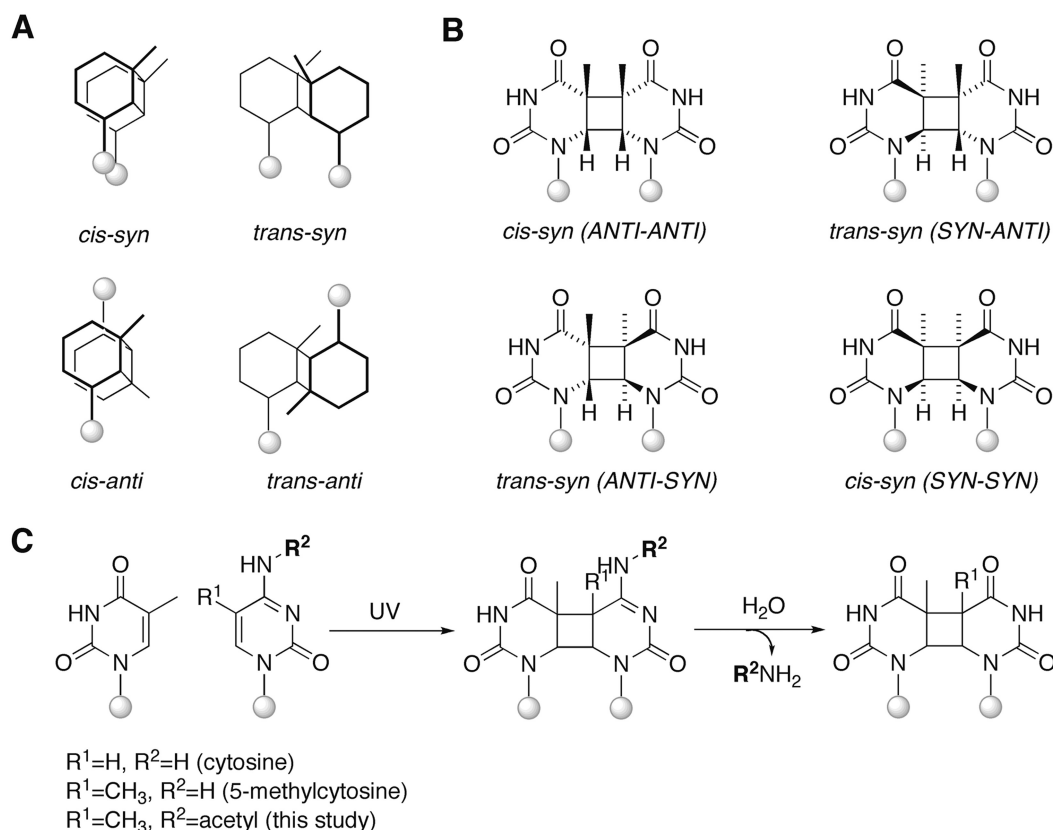


Figure 1. (A) Structures of CPDs formed between two pyrimidine bases. Small balls represent sugar moieties of nucleosides. (B) Four possible structures of CPDs formed between adjacent thymine bases viewed from the sugar-phosphate backbone. The conformations around the *N*-glycosidic bond of the 5'- and 3'-components are shown in parentheses next to the CPD structures. (C) Hydrolytic deamination of cytosine-containing CPDs.

yielded the *cis-anti* (15) and *trans-anti* (16) CPDs, respectively, with the latter formed in the loop region. These findings imply that the other types of CPD structures are biologically significant, in addition to the *cis-syn* CPD that has been exclusively studied.

The stereochemistry of the CPD formation between adjacent pyrimidine bases has been analyzed by using dinucleoside monophosphates over the past 30 years (17) (Figure 1B). UV irradiation of thymidylyl-(3'→5')-thymidine (TpT) reportedly yielded a mixture of the *cis-syn* and *trans-syn* CPDs in an 8:1 ratio (18), while UV irradiation of thymidylyl-(3'→5')-2'-deoxycytidine (TpC) yielded a complicated mixture of three stereoisomers, i.e. *cis-syn* with the ANTI-ANTI conformation around the *N*-glycosidic bonds of the 5'- and 3'-components, respectively, *trans-syn* with the SYN-ANTI conformation, and *trans-syn* with the ANTI-SYN conformation (Figure 1B), in a 4:3:1 ratio (19). In the case of TpC, it is well known that the exocyclic amino group at the C4 position of the cytosine base is subjected to rapid hydrolysis after the CPD formation, and the cytosine-containing CPD is converted to a uracil-containing one under physiological conditions (20–22) (Figure 1C), which causes a C→T transition mutation as a result of its reversal by UV or by the incorporation of dA by human DNA polymerase η (23). The rate constants of the deamination reaction are reportedly $2.5 \times 10^{-5} \text{ s}^{-1}$

and $6.5 \times 10^{-6} \text{ s}^{-1}$ for the *cis-syn* and *trans-syn* CPDs of TpC, respectively (24), which are similar to the values obtained for dCpT (25). In contrast, the attachment of a methyl group to the C5 position of the cytosine base ($\text{R}^1 = \text{CH}_3$ in Figure 1C) reduces the deamination rate of the cytosine-containing CPD (26,27), and thus the methylcytosine-containing CPD was utilized to analyze the biochemical properties of yeast DNA polymerase η (28).

Thus far, the CPD formation by a cytosine base modified at the amino group has not been reported. A modification at this position can influence the rate of hydrolytic deamination, because the basicity of the leaving group (R^2NH_2 in Figure 1C) as well as the electron density of the carbon atom that is attacked by a water molecule should be changed. Moreover, it is worthwhile to investigate whether the modification at this position affects the stereoselectivity of CPD formation. Here, we report the effect of the base modification on the cycloaddition reaction, by using thymidylyl-(3'→5')-2'-deoxy-*N*⁴-acetyl-5-methylcytidine. We found that UV irradiation of this compound in the presence of a photosensitizer exclusively afforded the cyclobutane thymine dimer (TT-CPD) with the *trans-syn* structure. The intermediate that putatively contained the acetyl amino group after the cycloaddition reaction was more stable in neutral solutions than under acidic

conditions, which indicated that the acetyl group facilitated the acid-catalyzed hydrolysis.

MATERIALS AND METHODS

General methods

Reagents for the DNA synthesizer were purchased from Applied Biosystems Japan (Tokyo, Japan) and Glen Research (Sterling, VA, USA). All of the other reagents and solvents were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). TLC analyses were carried out on Merck Silica gel 60 F254 plates, which were visualized by UV illumination at 254 nm. For column chromatography, Wakogel C-200 was used. HPLC analyses were performed on a Gilson or Shimadzu gradient-type analytical system equipped with a Waters 2996 or Shimadzu SPD-M10AVP photodiode-array detector. On both systems, a Waters μ Bondasphere C18 5 μ m 300 Å column (3.9 $\times$ 150 mm) was used, at a flow rate of 1.0 ml min⁻¹, with a linear gradient of acetonitrile in 0.1 M triethylammonium acetate (TEAA) (pH 7.0) generated over 20 min. ¹H, ¹³C and ³¹P NMR spectra were measured on a Varian INOVA 500 spectrometer. ¹H and ¹³C chemical shifts were calibrated with tetramethylsilane (TMS) and the ¹³C signals of the deuterated solvents (DMSO, 39.7 ppm; CDCl₃, 77.0 ppm) as internal references, respectively. ³¹P chemical shifts were calibrated with trimethylphosphate (TMP) as an external reference. 2D phase-sensitive NOESY and ROESY spectra were acquired with mixing times of 700 and 300 ms, respectively. High-resolution mass spectrometry was performed on a JEOL JMS-700 spectrometer with fast atom bombardment (FAB) ionization.

Synthesis of 3'-O-(tert-butyldimethylsilyl)-5'-O-(4,4'-dimethoxytrityl)-2'-deoxy-5-methylcytidine (2)

To a solution of 3'-O-(tert-butyldimethylsilyl)-5'-O-(4,4'-dimethoxytrityl)-4-triazolothymidine (**1**, 19.8 g, 27.8 mmol) (**29**) in acetonitrile (40 ml), 28% ammonia water (30 ml) was added, and then the flask was sealed with a rubber septum. After stirring for 3 h at room temperature, the ammonia was removed under reduced pressure on a rotary evaporator equipped with a vacuum pump, and the resultant solution was diluted with CHCl₃ (200 ml). The organic layer was washed with saturated aqueous NaHCO₃ (100 ml, twice) and brine (100 ml, once), and was dried over Na₂SO₄. The solvent was removed by evaporation *in vacuo*, and the residue was purified by column chromatography with a stepwise gradient of 0–5% methanol in CHCl₃. The collected fractions were evaporated to give the product as white foam. Yield 18.1 g (27.5 mmol, 99%).

Synthesis of 3'-O-(tert-butyldimethylsilyl)-2'-deoxy-5-methylcytidine (3)

A mixture of acetic acid (200 ml) and water (50 ml) was added to 3'-O-(tert-butyldimethylsilyl)-5'-O-(4,4'-dimethoxytrityl)-2'-deoxy-5-methylcytidine (**2**, 17.4 g, 26.5 mmol).

After 3.5 h, the reaction mixture was concentrated *in vacuo*. The residue was coevaporated with toluene and pyridine in turn, and then was purified by column chromatography with a stepwise gradient of 0–8% methanol in CHCl₃. The product was eluted in the 5–8% fractions, and the collected fractions were evaporated to give a white powdery product. Yield 7.82 g (22.0 mmol, 83%).

Synthesis of P-(2-cyanoethyl)thymidylyl-(3'→5')-3'-O-(tert-butyldimethylsilyl)-2'-deoxy-5-methylcytidine (4)

To a mixture of 3'-O-(tert-butyldimethylsilyl)-2'-deoxy-5-methylcytidine (**3**, 0.799 g, 2.25 mmol) and 5'-O-(4,4'-dimethoxytrityl)thymidine 3'-(2-cyanoethyl)-N, N-diisopropylphosphoramidite in anhydrous acetonitrile (10 ml), a solution of benzimidazolium triflate (3.62 g, 13.5 mmol) in anhydrous acetonitrile (60 ml) was added. After stirring the solution for 5 h at room temperature, saturated aqueous NaHCO₃ (8 ml) was added. The reaction mixture was diluted with CHCl₃ (100 ml), and was washed with saturated aqueous NaHCO₃ (70 ml, twice) and brine (70 ml, once). The organic layer was dried over Na₂SO₄, and then was evaporated *in vacuo*. The residue was dissolved in a 0.11 M iodine solution (THF/pyridine/water = 40/9/1, v/v/v, 80 ml). After stirring for 1 h, a 1 M solution of aqueous Na₂S₂O₃ (100 ml) and ethyl acetate (150 ml) were added to the reaction mixture. The organic layer was separated and dried over Na₂SO₄. After evaporation, the residue was co-evaporated with toluene, and was purified by column chromatography with a stepwise gradient of 0–7% methanol in CHCl₃. The tritylated product was eluted in the 3–6% fractions, and the collected fractions were concentrated to dryness to obtain a white foamy product. This product was subsequently dissolved in 80% aqueous acetic acid (200 ml). After 2.5 h, the acetic acid and water were removed *in vacuo*. The residue was co-evaporated with toluene, and was purified by column chromatography with a stepwise gradient of 0–12% methanol in CHCl₃. The desired product was eluted in the 7–11% fractions, and they were concentrated to dryness. The obtained product was dissolved in 5% methanol in CHCl₃ (5 ml), and the solution was dropped into a mixture of hexane and diethyl ether (1/1, v/v, 100 ml) to give a white powder. Yield 1.09 g (1.53 mmol, 68%).

Stereoselective formation of the *trans-syn* TT-CPD

To protect the amino group, acetic anhydride (113 μ l, 1.20 mmol) was added to a solution of P-(2-cyanoethyl)thymidylyl-(3'→5')-3'-O-(tert-butyldimethylsilyl)-2'-deoxy-5-methylcytidine (**4**, 0.712 g, 1.00 mmol) in anhydrous DMF (33.6 ml). The reaction was monitored by HPLC, and after 70 h, the starting material was completely consumed and converted to the acetylated product **5**. Water and ethyl acetate were added to the resultant mixture, and the organic layer was washed with water and evaporated to dryness. The residue was dissolved in 50% aqueous acetonitrile (1 l). After a nitrogen purge for 10 min, acetophenone (1 ml, 8 mmol) was added, and then the solution was irradiated in a Pyrex immersion well

apparatus fitted with a 450 W high-pressure mercury lamp (UM-452; Ushio, Tokyo, Japan) in a 4°C water bath (NCB-2200; EYELA, Tokyo, Japan). Aliquots (500 µl) of the reaction mixture were evaporated *in vacuo*, and the residues were dissolved in 50% aqueous acetonitrile (500 µl). These samples, collected at appropriate intervals, were analyzed by HPLC using a 30–39% acetonitrile gradient generated over 20 min. After 8 h irradiation, the solution was concentrated to dryness, and the residue was purified by column chromatography with a stepwise gradient of 0–6% methanol in CHCl₃. The 4–6% fractions were collected and evaporated to dryness to give a white product. The diastereomers were separated on a column (1.5 cm i.d. × 47 cm) of alkylated silica gel (Preparative C18 55–105 µm, Waters Corporation, Milford, MA, USA) with a linear gradient of 35–60% aqueous acetonitrile generated over 400 min, using a Bio-Rad BioLogic LP system. The appropriate fractions for collection were determined by HPLC. The solutions containing each diastereomer were evaporated to give a white product (**6a** and **6b**). Total yield 393 mg (551 µmol, 55%). **6a** (peak iii–a in Figure 2), Yield 164 mg (230 µmol); **6b** (peak iii–b in Figure 2), Yield 229 mg (321 µmol).

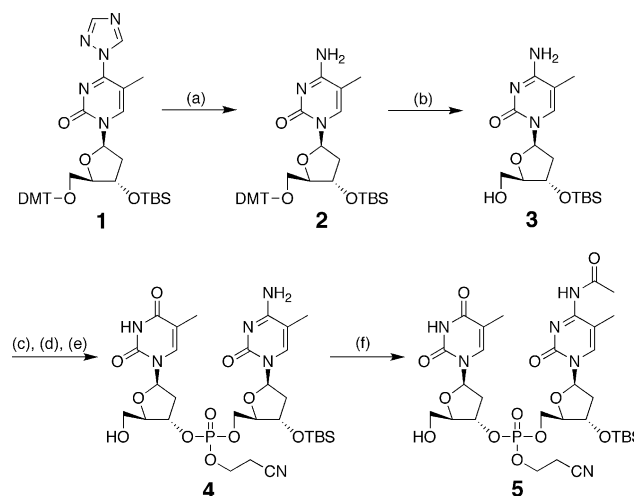
Analysis of the pH and temperature dependence of the hydrolysis reaction

Acetic anhydride (2.8 µl, 30 µmol) was added to a solution of *P*-(2-cyanoethyl)thymidylyl-(3'→5')-3'-*O*-(*tert*-butyldimethylsilyl)-2'-deoxy-5-methylcytidine (**4**, 18 mg, 25 µmol) in anhydrous DMF (850 µl), and the reaction mixture was stirred for 70 h. Water and ethyl acetate were added to the mixture, and the organic layer was separated, washed with water, and evaporated to dryness. The residue was co-evaporated with acetonitrile, and was dissolved in 50% aqueous acetonitrile (25 ml). After a 5-min nitrogen purge, acetophenone (23.4 µl, 200 µmol) was added to the solution, which was then placed in a bottle. This bottle was attached to the Pyrex jacket for a 450 W high-pressure mercury lamp, and was immersed in a 4°C water bath. Using this apparatus, the solution was irradiated for 1 h. Aliquots (3 ml) of the reaction mixture were mixed with 0.4 M phosphate buffer (1.5 ml) and acetonitrile (1.5 ml), and the buffered solutions were kept at 4, 35, or 50°C. At appropriate intervals, aliquots (40 µl) of the solutions were collected in a plastic tube, and the tubes were immediately placed in dry ice/EtOH. The frozen samples were kept at –80°C until the HPLC analysis was performed. The pH values of the solutions were measured on a Mettler Toledo SevenEasy S20 pH meter, calibrated at pH 6.86, 4.01 and 1.68.

RESULTS

Preparation of protected thymidylyl-(3'→5')-2'-deoxy-5-methylcytidine

To investigate the cycloaddition reaction between thymine and 5-methylcytosine in the presence of a base modification, we synthesized *P*-(2-cyanoethyl)thymidylyl-(3'→5')-*N*⁴-acetyl-3'-*O*-*tert*-butyldimethylsilyl-2'-deoxy-5-methyl



Scheme 1. Synthesis of TpdmC^{ac}. Reagents and conditions: (a) NH₃/H₂O, CH₃CN, (b) 80% CH₃COOH (c) 5'-*O*-(4,4'-dimethoxytrityl)thymidine 3'-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite, BIT/CH₃CN, (d) I₂, H₂O/THF, (e) 80% CH₃COOH and (f) acetic anhydride/DMF.

cytidine (TpdmC^{ac}, **5**), as shown in Scheme 1. Since 5-methylcytosine is converted to thymine by hydrolytic deamination, 2'-deoxy-5-methylcytidine was employed in this study, instead of 2'-deoxycytidine, to facilitate the determination of the stereochemistry of the resultant CPD, which has the same structure as that derived from two thymine bases, by HPLC and NMR analyses. We chose the acetyl group for the modification of the exocyclic amino function of 5-methylcytosine, because this group is relatively small and lacks aromatic rings, which absorb the light energy and disturb the photoreaction. Since the acetyl group is an electron-withdrawing functional group, it was expected that the hydrolysis of the amino group in this type of CPD would occur more rapidly than that of the unmodified amino group.

First, protected 2'-deoxy-5-methylcytidine (**2**) was synthesized from a derivative of 4-triazolothymidine (**1**) (29), by a treatment with ammonia water. To avoid the side reaction at the coupling step, we tried to protect the amino function of the 5-methylcytosine with the acetyl group at this step, but we found that the *N*-acetyl group was easily removed at the detritylation step before the coupling with the 5'-component. We therefore planned to introduce the acetyl group later, as shown in Scheme 1, and according to this procedure, the 5'-hydroxyl group was deprotected to yield compound **3**. In the following coupling reaction between the thymidine phosphoramidite and the 3'-OH-protected 5-methylcytidine (**3**), benzimidazolium triflate (BIT) was used as an alternative activator to tetrazole, to ensure the *O*-selective reaction of the activated phosphoramidite (30–32). After this coupling reaction, the oxidation of the internucleoside phosphite linkage and the detritylation at the 5'-end afforded a dinucleoside monophosphate (**4**) to use for the *N*-acetylation followed by the photoreaction.

Cycloaddition between thymine and *N*⁴-acetylated 5-methylcytosine

To prepare the base-modified dinucleoside monophosphate for the analysis of CPD formation, partially-protected thymidylyl-(3'→5')-2'-deoxy-5-methylcytidine (**4**) was treated with acetic anhydride in anhydrous DMF. However, the acetyl group was removed during the purification of the product, by either the silica-gel or reversed-phase column chromatography. We therefore used the acetylated product without chromatographic purification for the cycloaddition reaction. To ensure the complete conversion of the starting material, the acetylation reaction was monitored by HPLC (Supplementary Figure S1A). An analysis of the reaction mixture revealed that the starting material (**4**, Rt 8.2 min, peak i in Supplementary Figure S1A) was consumed completely after ~70 h, and was converted to a new product (Rt 9.7 min, peak ii) with characteristic absorption maxima at 249.0 and 308.3 nm (trace b in Supplementary Figure S1B), in contrast to that of the starting material at 270.3 nm (trace a). In addition, high-resolution mass spectrometry of the product revealed that its molecular mass was m/z 755.2849 ($[M+H]^+$, calculated for $C_{31}H_{48}N_6O_{12}PSi$, 755.2838). These results indicated that a single acetyl group was

attached to 5-methylcytosine, and that the desired compound (**5**) was successfully obtained.

The above reaction mixture was concentrated *in vacuo*, and the residue was dissolved in 50% aqueous acetonitrile. This solution, containing the acetylated product (**5**), was irradiated with a 450 W Pyrex-filtered high-pressure mercury lamp at 5°C in the presence of acetophenone, and aliquots of the reaction mixture obtained at appropriate intervals were analyzed by HPLC (Figure 2). The starting material (**5**, Rt 9.7 min, peak i) was converted to new products that gave a pair of peaks with longer retention times of 15.5 min and 21.7 min (peaks ii-a and ii-b, respectively). Upon irradiation, the areas of peaks ii-a and ii-b gradually decreased, and other peaks with retention times of 11.9 min and 16.4 min (peaks iii-a and iii-b, respectively) emerged. After irradiation for 6 h (trace e in Figure 2), the peak ii pair completely disappeared, and the second pair of peaks (iii-a and iii-b) were detected as the main products. The UV absorption spectrum of peak ii-a was identical to that of ii-b (trace b in Supplementary Figure S2), and the same held true for peaks iii-a and iii-b (trace c in Supplementary Figure S2). A comparison of these spectra indicated that the cycloaddition reaction between thymine and acetylated 5-methylcytosine successfully occurred, because neither peaks ii-a/b nor iii-a/b exhibited absorption at wavelengths longer than 280 nm. Interestingly, the absorption spectrum of peak ii-a/b was different from that of peak iii-a/b, and peak ii-a/b had a shoulder in the region of 230–270 nm (traces b and c in Supplementary Figure S2). These results indicated that the second products yielding peaks iii-a and iii-b were produced from the intermediates that yielded peaks ii-a and ii-b, by a change in the chemical structure of the base moiety.

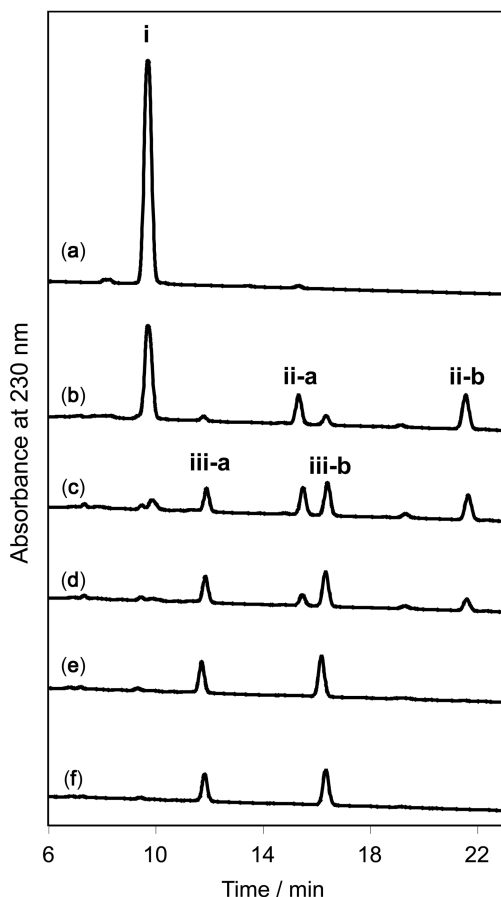


Figure 2. HPLC analysis of the photosensitized cycloaddition of *N*-acetylated dinucleoside monophosphate (**5**), monitored at 230 nm. Aliquots of the reaction mixtures were analyzed after UV irradiation for (a) 0, (b) 1.5, (c) 3, (d) 4.5, (e) 6 and (f) 8 h.

Characterization of the products obtained by the cycloaddition reaction of TpdMC^{ac}

To analyze the structural change, the final products yielding peaks iii-a and iii-b were purified by silica gel column chromatography, and the resultant mixture was irradiated with UV light to confirm the presence of the cyclobutane ring by photoreversal (traces c and d in Supplementary Figure S1A). This irradiation converted the two peaks to a new product (peak iii in Supplementary Figure S1A) with a retention time of 13.6 min and an absorption maximum at 266.8 nm (trace c in Supplementary Figure S1B), which did not coincide with those of the starting material.

Next, the compounds that yielded peaks iii-a and iii-b in Figure 2 were separated by reversed-phase column chromatography to determine their chemical structures. High-resolution mass spectrometry revealed that these compounds had the same molecular mass of m/z 714.2555, which was identical to that of partially-protected TpT [*P*-(2-cyanoethyl)thymidylyl-(3'→5')-3'-*O*-*tert*-butyldimethylsilylthymidine] (calculated m/z 714.2572), indicating that they contained the CPD derived from two thymines. To determine their stereochemistry, the NMR spectra of these compounds were measured. 2D ROESY experiments performed with a

mixing time of 300 ms (Supplementary Figure S3) revealed that both of them had characteristic ROE signals between TpH6 and TpH1', between CH₃ of the base moiety and H6, and between pTH6 and pTH2', in which Tp and pT represent the 5'- and 3'-components of the CPD, respectively, although the TpH6 and pTH6 resonances (δ 4.06 ppm) of the compound responsible for peak iii-a were apparently completely overlapped (Supplementary Figure S3A). These ROE signals indicated that the CPD in these compounds had the *trans-syn* structure with the SYN-ANTI conformation.

For further characterization, the 2-cyanoethyl and *tert*-butyldimethylsilyl (TBS) groups in the compounds yielding peaks iii-a and iii-b were removed, by treatments with ammonia water and triethylamine trihydrofluoride, respectively, and the deprotected materials were combined and analyzed by HPLC (trace a in Figure 3A). After deprotection, a single peak was detected at a retention time of 8 min, and the product from the compound yielding peak iii-a was identical to that from peak iii-b. This means that the two compounds (peaks iii-a and iii-b) obtained by cycloaddition of Tpdmc^{ac} (**5**) were the diastereomers caused by the chirality of the internucleoside phosphotriester, and these compounds were assigned to compound **6** in Scheme 2. These diastereomers were deprotected and analyzed by ¹H NMR, and all of the proton resonances were obviously identical to those of the dinucleoside monophosphate containing the *trans-syn* TT-CPD with the SYN-ANTI conformation reported previously (17,33). From these results, we concluded that Tpdmc^{ac} undergoes the photosensitized cycloaddition reaction in a stereo-controlled manner to yield the *trans-syn* CPD. The chemical structure of compound **6** strongly suggested that the compounds yielding peaks ii-a and ii-b, which were detected as intermediates, were diastereomers containing the *trans-syn* CPD with the SYN-ANTI conformation formed between thymine and *N*⁴-acetyl-5-methylcytosine, and that these intermediates underwent hydrolysis to yield the *trans-syn* TT-CPD (Scheme 2).

To compare the diastereoselectivity of the CPD formation, the cycloaddition reaction of *P*-(2-cyanoethyl)-thymidylyl-(3'→5')-thymidine 3'-2-oxopentanoate, which was an intermediate for the preparation of the building block of the *cis-syn* TT-CPD [compound **3** in (34)], was tested under the same irradiation conditions. The reaction mixture was concentrated to dryness, and the residue was treated with ammonia water to remove the protecting groups. In the HPLC analysis of the deprotected product, two peaks were detected as the main products (trace b in Figure 3A), and were assigned to the *cis-syn* (ANTI-ANTI) (peak i) and *trans-syn* (SYN-ANTI) (peak ii) TT-CPDs (18). This result demonstrated that the photosensitized cycloaddition of Tpdmc^{ac} occurred in a highly stereoselective manner.

Since the 5-methyl group stabilizes the amino function in the CPD against hydrolysis to some extent and produces a mixture of the thymine-5-methylcytosine and thymine-thymine CPDs after photosensitized cycloaddition, thymidylyl-(3'→5')-2'-deoxycytidine derivatives without this methyl group [**7**, which was used in our

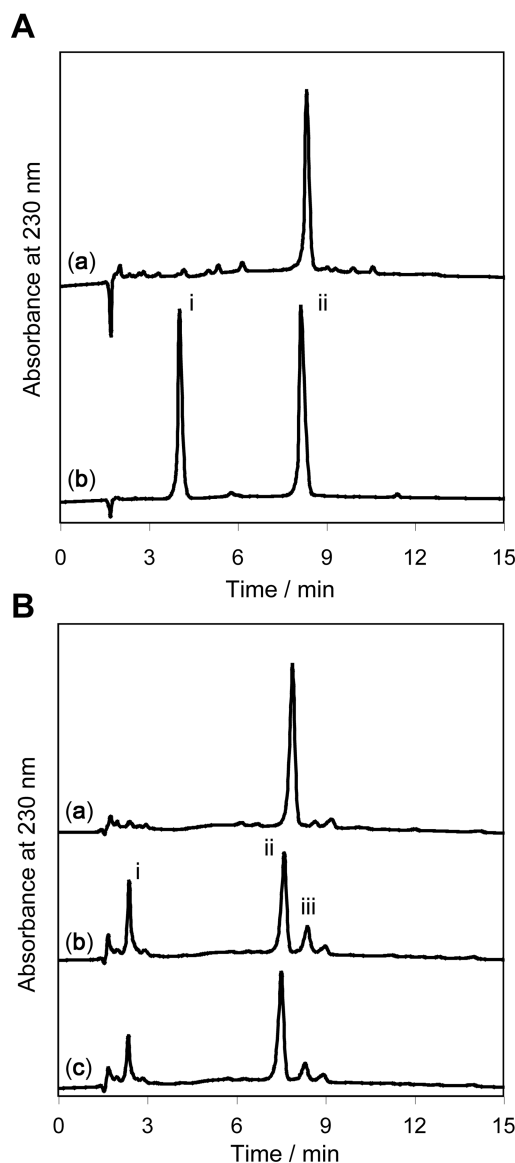
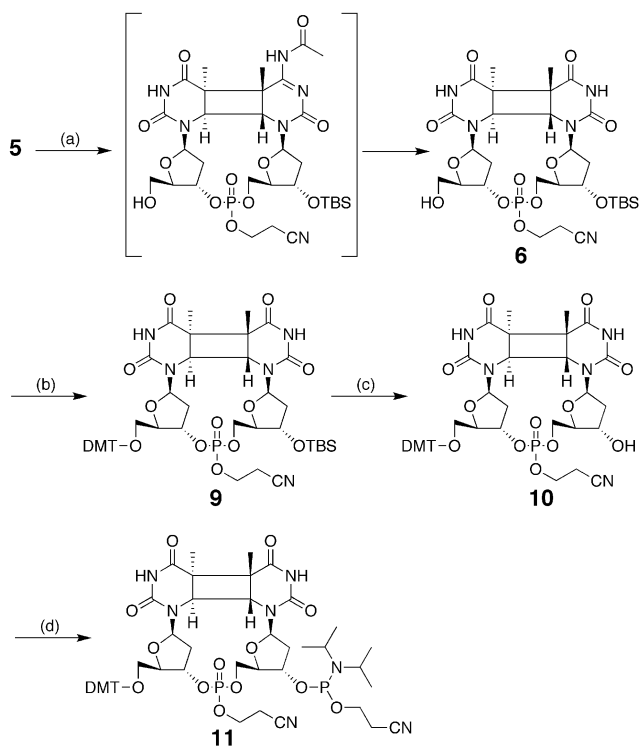
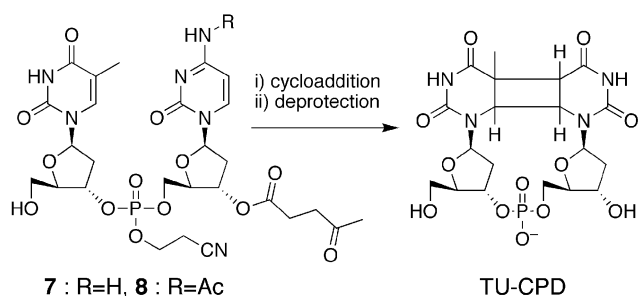


Figure 3. (A) HPLC analysis of the deprotected TT-CPDs obtained from (a) compound **5** and (b) partially-protected TpT [*P*-(2-cyanoethyl)thymidylyl-(3'→5')-thymidine 3'-2-oxopentanoate]. Peaks i and ii were assigned to the *cis-syn* TT-CPD (ANTI-ANTI) and the *trans-syn* TT-CPD (SYN-ANTI), respectively. (B) HPLC analysis of the deprotected TU-CPDs obtained from (a) compound **7** and (b) compound **8**. Peaks i, ii, and iii were assigned to the *cis-syn* TU-CPD (ANTI-ANTI), the *trans-syn* TU-CPD (SYN-ANTI), and the *trans-syn* TU-CPD (ANTI-SYN), respectively. (c) A co-elution experiment of (a) and (b). The analysis was performed on a reversed-phase column with a linear gradient of 0–10% acetonitrile in 0.1 M TEAA for 20 min.

previous study (35), and **8** in Scheme 3] were used to analyze the effect of the *N*-acetyl group on the stereochemistry of the CPD formation. Without the 5-methyl group, the amino group was expected to be hydrolyzed rapidly, to generate only the thymine-uracil CPD (TU-CPD). After the photosensitized cycloaddition of these compounds, followed by a treatment with hot ammonia water, the crude samples were analyzed by HPLC, as shown in Figure 3B. Cycloaddition of



Scheme 2. Synthesis of the phosphoramidite building block of the *trans-syn* TT-CPD. Reagents and conditions: (a) UV (>280 nm), acetophenone/H₂O, CH₃CN, (b) DMTCl/pyridine, (c) TEA·3HF/THF and (d) 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, DIPEA/THF.



Scheme 3. Preparation of the dinucleoside monophosphate containing the TU-CPD.

compound **8** afforded a single product (trace a in Figure 3B), and NMR analysis of the isolated product revealed that it had the *trans-syn* TU-CPD with the SYN-ANTI conformation. On the other hand, three peaks were detected for the reaction mixture that started from compound **7** (trace b in Figure 3B). These products were assigned to the *cis-syn* (ANTI-ANTI) (peak i), *trans-syn* (SYN-ANTI) (peak ii), and *trans-syn* (ANTI-SYN) (peak iii) TU-CPDs, by coelution with the product from compound **8** or the authentic *cis-syn* TU-CPD (**23**). The ratio of these products was 6:7:2, respectively, and the order of elution was identical to that reported previously (**24**). These results clearly showed that the modification of the cytosine amino function with

the acetyl group afforded the stereoselectivity of the CPD formation even though the 5-methyl group was not present.

pH and temperature dependence of the intermediate hydrolysis

The cycloaddition of Tpd^mC^{ac} (**5**) afforded the *trans-syn* TT-CPD (**6**) through hydrolysis of the intermediate. For comparison, the cycloaddition reaction in the non-acetylated dinucleoside monophosphate (**4**) was examined. The products obtained from compound **4** by UV irradiation for 6 h were sufficiently stable, and were reversed to the intact bases by the second UV irradiation at 254 nm. This result agreed well with that described in a previous report (**28**), and supported the aforementioned idea that the intermediates yielding peaks ii-a and ii-b in Figure 2 had the acetylamino group.

To evaluate the effect of the acetyl group on the hydrolysis of the intermediate, kinetic analyses were performed under several pH conditions. After UV irradiation of compound **5** for 1 h, when the TT-CPD (**6**) was not formed yet, the solution was mixed with phosphate buffer containing 50% acetonitrile, and was kept at 4°C. Aliquots obtained at appropriate intervals were analyzed by HPLC, and the total peak areas of the intermediates were plotted on a logarithmic vertical axis against the incubation time (Figure 4A). At pH 6.9, only a slight decrease of the intermediate was observed during 6 h, and after 10 days, ~60% of the intermediate was converted to the TT-CPD (data not shown). In contrast, the reaction was faster at lower pH values. It should be noted that the areas of the two intermediate peaks (ii-a and ii-b) decreased at the same rate, supporting the idea that these two intermediates were diastereomers bearing the same CPD structure, which were converted to the corresponding *trans-syn* TT-CPD diastereomers (**6**). The decrease in the peak areas could be approximated well by a pseudo-first order equation, and the observed reaction rate constants (k_{obs}) were obtained. These values and the half-lives are summarized in Table 1. The results indicated that the conversion of the intermediate to the TT-CPD strongly depends on the pH value of the solution.

Next, the incubation temperatures were raised to 35 and 50°C, to investigate the temperature dependence of the conversion reaction. The HPLC analysis of the pH 5.0 solution revealed the acceleration of the conversion rate by the increased incubation temperature (Figure 4B). This temperature-dependent acceleration was also observed for the neutral solution (Supplementary Figure S4). From an Arrhenius plot (Supplementary Figure S5), the activation energy (E_a) of this conversion at pH 5.9 was calculated to be 29.5 kJ mol⁻¹.

Phosphoramidite building block of the *trans-syn* TT-CPD and its incorporation into oligonucleotides

From the diastereomers containing the *trans-syn* TT-CPD (**6**), a phosphoramidite building block (**11**) for its incorporation into oligonucleotides was prepared, as shown in Scheme 2. Compounds **9a**, **9b**, **10a** and **10b**, in which a and b represent the diastereomers caused by the chirality at

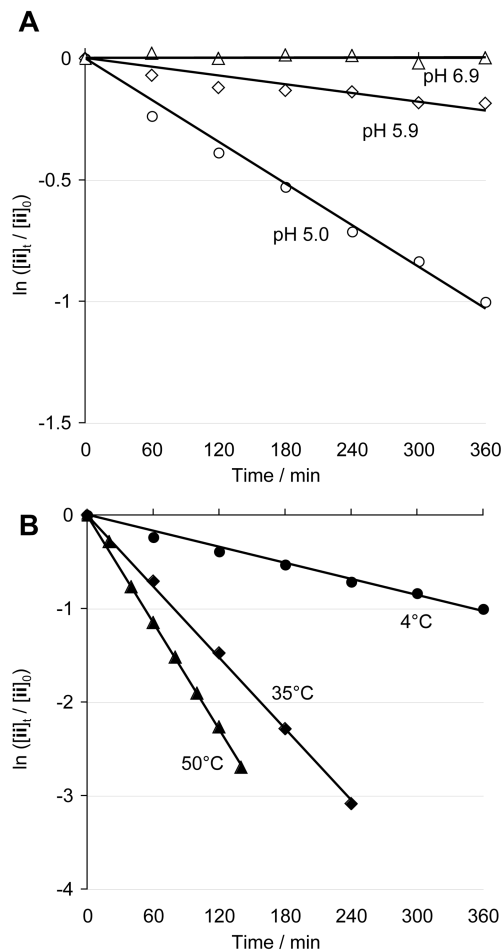


Figure 4. (A) pH dependence of the conversion of the intermediate to the TT-CPD (6) at 4°C. (B) Temperature dependence of the same reaction at pH 5.0. In both cases, $[ii]_t$ and $[ii]_0$ represent the total areas of peaks ii-a and ii-b in Figure 2 at t min and 0 min, respectively.

Table 1. Observed rate constants and half-lives of the conversion of the intermediate to TT-CPD

	Observed rate constant (k_{obs})/min ⁻¹			Half-life ($\tau_{1/2}$)/h		
	4°C	35°C	50°C	4°C	35°C	50°C
No buffer ^a	2.11×10^{-3}	4.23×10^{-3}	7.27×10^{-3}	5.5	2.7	1.6
pH 5.0	2.86×10^{-3}	1.27×10^{-2}	1.91×10^{-2}	4.0	0.9	0.6
pH 5.9	6.02×10^{-4}	2.07×10^{-3}	3.79×10^{-3}	19.2	5.6	3.0
pH 6.9	$<6.15 \times 10^{-5}$	2.76×10^{-4}	1.31×10^{-3}	>188	41.9	8.8

^aThe pH value was found to be acidic (pH 5.0 at 4°C).

the phosphorus atom, were characterized by high-resolution mass spectrometry and NMR spectroscopy (Supplementary Data). Using this building block, an oligonucleotide, d(CGCGAATTGCGCCC), where underlined TT represents the *trans-syn* TT-CPD, was successfully synthesized on a DNA synthesizer. This oligonucleotide was characterized by a photoreversal experiment and MALDI-TOF mass spectrometry (Supplementary Figures S6 and S7, respectively).

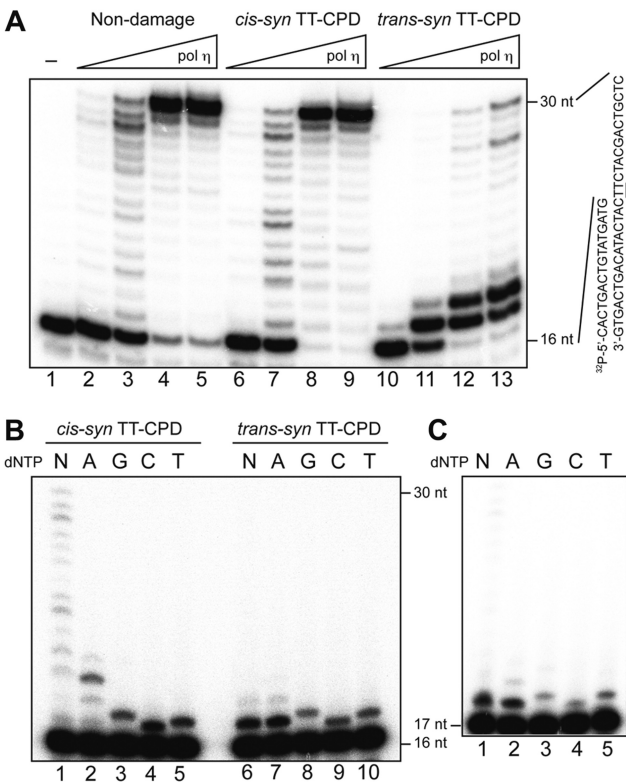


Figure 5. (A) Translesion synthesis by hPol η. The ³²P-labeled 16-mer primer and the 30-mer templates containing the intact TT (lanes 1–5), the *cis-syn* TT-CPD (lanes 6–9), and the *trans-syn* TT-CPD (lanes 10–13) were incubated with increasing amounts of hPol η (1.6, 16, 64, 128 fmol) at 37°C for 15 min. The sample for lane 1 contained no enzyme. (B) Selectivity of nucleotide incorporation by hPol η opposite the 3'-components of the *cis-syn* (lanes 1–5) and *trans-syn* (lanes 6–10) CPDs. The enzyme amount was 4 fmol. The reaction mixtures were incubated for 3 min in the presence of the four dNTPs (lanes 1 and 6) or the indicated dNTP (lanes 2–5 and 7–10). (C) Selectivity of nucleotide incorporation by hPol η opposite the 5'-component of the *trans-syn* CPD. The ³²P-labeled 17-mer primer and the 30-mer template containing the *trans-syn* CPD were incubated with 8 fmol of hPol η for 15 min in the presence of the four dNTPs (lane 1) or the indicated dNTP (lanes 2–5).

Translesion synthesis of the *trans-syn* CPD by human DNA polymerase η

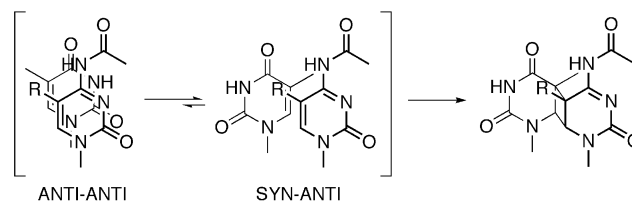
We also synthesized a longer oligonucleotide containing the *trans-syn* TT-CPD, d(CTCGTCAGCATCTTCATCATACAGTCAGTG) (tsCPD 30-mer), and used it for the analysis of translesion synthesis (TLS) by human DNA polymerase η (hPol η). Primer extension experiments were carried out, using tsCPD 30-mer and a 30-mer containing the *cis-syn* TT-CPD (34) as templates (Figure 5). Although hPol η catalyzed extension of the primer past the *cis-syn* CPD with an efficiency similar to that observed for the non-damaged template, the chain elongation was blocked after incorporation of a nucleotide opposite the 3'-component of the *trans-syn* CPD (lane 11 in Figure 5A). At higher enzyme concentrations, the second nucleotide was incorporated opposite the 5'-component (lanes 12 and 13). The selectivity of nucleotide incorporation opposite each component of the *trans-syn* CPD was determined by using only one of the nucleotides

(Figure 5B and C). In contrast to the accurate bypass of the *cis-syn* CPD by hPol η (lanes 1 and 2 in Figure 5B), efficient incorporation of the second dAMP did not occur in the case of the *trans-syn* CPD (lane 7 in Figure 5B). As for the second nucleotide, dAMP was the most likely candidate to be incorporated opposite the 5'-component of the lesion (Figure 5C). These results showed that the 5'-component of the *trans-syn* CPD, which is in the SYN conformation around the *N*-glycosidic bond, could not be bypassed by hPol η .

DISCUSSION

In this study, we investigated the intramolecular cycloaddition of an *N*⁴-acetylcytosine-containing dinucleoside monophosphate, to gain new insights into the effect of the base modification on the stereochemistry of the CPD formation and the hydrolytic deamination. We found that the photosensitized cycloaddition of TpdM^{ac} (**5**) resulted in the stereoselective formation of the *trans-syn* TT-CPD with the SYN-ANTI conformation. The conversion from 5-methylcytosine to thymine was caused by the hydrolysis of saturated *N*⁴-acetyl-5-methylcytosine after the CPD formation. The kinetic analysis of this hydrolysis reaction demonstrated that the reaction depends on both pH and temperature, as reported earlier (24). The intermediate with the acetylamino group was found to be more stable in a neutral solution than in an acidic solution. This result implies that protonation of the N3 imino function of the 5-methylcytosine derivative triggers the hydrolysis, followed by rapid dissociation of acetamide. An analysis of the temperature dependence revealed that the activation energy of the hydrolysis reaction at pH 5.9 was 29.5 kJ mol⁻¹, which was comparable to that reported for a dinucleoside monophosphate of the *trans-syn* TC-CPD (69.7 kJ mol⁻¹) (24). Since an increase in the ionic strength of the solution reportedly accelerates the hydrolytic deamination (36), these activation energy values cannot be compared to each other. Nonetheless, the lower activation energy obtained in this study indicates that the hydrolysis of the intermediate is accelerated by the acetyl group, even in the presence of the C5 methyl group that stabilizes the amino function against hydrolysis.

The cycloaddition of the *N*-acetylated compounds **5** and **8**, with and without the C5 methyl group, respectively, occurred in a highly stereoselective manner to yield the common CPD structure, namely *trans-syn* with the SYN-ANTI conformation. An essential question is how the acetyl group regulates the stereoselectivity in the CPD formation. Since the obtained CPD structure is determined by the alignment of the two bases when the reaction occurs, a possible explanation is that the stereoselective formation of the TT- and TU-CPDs in the presence of the *N*⁴-acetyl modification is caused by steric hindrance by the acetyl group. It may be unfavorable for the two bases to adopt the *cis* structure when one of them bears the acetyl group (Scheme 4). In contrast, when the two bases are present in the *trans* structure, the acetyl group does not hinder the two C5–C6 double bonds from approaching each other to form the cyclobutane



Scheme 4. A possible mechanism for the stereoselective formation of the *trans-syn* CPD.

ring. This relative positioning of the two bases for the cycloaddition probably allows the stereoselective formation of the *trans-syn* CPD.

In contrast to the abundant physicochemical and biochemical studies on the CPD, the stereo-controlled formation of the CPD has not been investigated. In the preparation of a phosphoramidite building block for the conventional solid-phase synthesis of CPD-containing oligonucleotides, it has been pointed out that the photosensitized cyclobutane formation in a properly-protected dinucleoside monophosphate usually yields a mixture of more than four diastereomers, due to the presence of two or three CPD structures and the chirality of the internucleoside phosphotriester (37), and laborious manipulation to separate these products is required at this step. Taylor and co-workers addressed this problem by using a diastereomerically pure phosphoramidite at the coupling step, which could exclude the formation of diastereomers caused by the phosphorus chirality (37), or by separating the *cis-syn* and *trans-syn* CPDs after deprotection of the oligonucleotides (38). Carell and co-workers used a CPD-like lesion in which the internucleoside phosphate was replaced with a formacetal linkage (39,40) or a compound without a linkage between the sugar moieties (41,42). Both of these approaches sought to resolve the problem by removing the chiral phosphorus atom. On the other hand, regulation of the stereochemistry in the cyclobutane ring formation is straightforward but challenging, and the present study supports the possibility of a new strategy. In this study, the *trans-syn* TT- and TU-CPDs were obtained by the cycloaddition of dinucleoside monophosphates containing *N*⁴-acetyl-5-methylcytosine and *N*⁴-acetylcytosine, respectively. It should be noted that the *trans-syn* TU-CPD was not obtained in our previous study on the synthesis of oligonucleotides containing the *cis-syn* TU-CPD (23). Although the starting materials and the products in the cycloaddition reaction are the diastereomers caused by the internucleoside phosphotriester, this reaction is still advantageous, because the separation of these diastereomers is not essential for the oligonucleotide synthesis. The chiral phosphotriester is finally converted to a non-chiral phosphodiester by deprotection.

One of the oligonucleotides containing the *trans-syn* TT-CPD synthesized in this study was used for the analysis of the TLS by hPol η , which is encoded by the *XPV* (xeroderma pigmentosum variant) gene and is responsible for the error-free replication past the *cis-syn* TT-CPD (43,44). We found that hPol η was almost

stalled after incorporation of dAMP opposite the 3'-component of the *trans-syn* CPD. Since the base orientation of the 5'-component is fixed in the SYN conformation, there may be a steric hindrance when this lesion enters the active site of the enzyme, and the 5'-component cannot make a base pair with the incoming nucleotide even if it can fit the active site. This is an example showing that the stereochemistry of a biomolecule affects its biological property.

In conclusion, we investigated the cycloaddition reaction between thymine and *N*-acetylated 5-methylcytosine, and found that the reaction proceeded in a diastereoselective manner to yield the *trans-syn* CPD with the SYN-ANTI conformation. In addition, the acetylamino group was hydrolyzed more rapidly than the unmodified amino group. These findings provided an efficient method for the synthesis of oligonucleotides containing this type of UV lesion. Further studies, including experiments using other types of modifications, are required to elucidate the mechanism that generates this stereoselectivity.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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