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Salicylic Acid as a Photosensitizer for Thymidine Dimerization Induced by UV

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When a neutral solution of a nucleoside mixture was irradiated with UV light having wavelength longer than 300 nm, addition of salicylic acid to the solution greatly accelerated the reaction of thymidine. The UV light irradiation of thymidine solution in the presence of salicylic acid resulted in four major product peaks in HPLC. All the products were identified as isomers of cyclobutane thymidine dimers by MS and NMR. The cyclobutane thymidine dimers were generated from thymidine almost exclusively. UV irradiation with the longer wavelength of 350 nm induced almost no reaction. The results indicate that salicylic acid is a photosensitizer for thymidine dimerization excited by UV light of wavelength 300 to 350 nm.

Key words salicylic acid; thymidine dimer; UV light; photosensitizer

Introduction

Exposure to UV light from the sun is associated with the occurrence of skin cancer in humans.¹⁾ UV light directly induces various forms of DNA damage including bipyrimidine photoproducts, cytosine hydrates, and 8-oxo-dGuo.^{2,3)} Among these, cyclobutane thymine dimers were found to be the major photoproducts generated by artificial UV light and sunlight within cellular DNA.²⁻⁴ In the UV light reaction of thymidine (dT), a free nucleoside, six configurationally-distinct forms of cyclobutane thymidine dimer are formed.⁵⁾ The cys-sin and trans-anti isomers exist as single molecules, whereas both of the trans-syn and cis-anti isomers exist as enantiomeric pairs. In the presence of a photosensitizer, UV light with longer wavelength and visible light can indirectly induce DNA damage. Many studies have shown that ketone photosensitizers such as acetone and acetophenone enhance the formation of thymidine dimers from dT through energy transfer.²⁾ Salicylic acid (SA) is a good agent for peeling, since it can exfoliate the corneum.⁶⁾ Many cosmetic products, including shampoos, soaps, lipsticks, and solution serums for skin care, include SA as an ingredient at concentrations up to 3% (ca. 200 mM).⁷⁾ It is reported that SA is not a photosensitizer and does not show phototoxycity.^{7,8)} In the present study, we investigated the effect of SA on the reaction of dT with UV light, and report here that salicylic acid is a photosensitizer of the reaction of dT with UV from 300 to 350nm, resulting in cyclobutane thymidine dimers almost exclusively.

Results and Discussion

A mixed solution of nucleosides (2'-deoxycytidine (dC), 2'-deoxyguanosine (dG), dT, and 2'-deoxyadenosine (dA); 100 μ M each) with SA in 100 mM potassium phosphate buffer at pH 7.4 was irradiated with UV light from a high-pressure mercury lamp through a 300 nm longpass filter at a temperature of 37°C. The reaction mixture was analyzed by reversed phase (RP) HPLC. The nucleoside concentrations were determined by absorbance areas of HPLC detected at 260 nm. In the presence of 1 mM SA, the concentration of dT greatly decreased as the irradiation time increased, whereas that of dG slightly decreased and those of dC and dA did not change, as shown in Fig. 1A. Figure 1B shows the SA dose-dependent changes on concentrations of nucleosides when the nucleoside solution with 0–2 mM SA was irradiated with UV light for 20 min. The concentration of dT decreased with increasing SA dose up to 1 mM, whereas for 1–2 mM SA, the concentration of nucleosides was unchanged. Table 1 shows the effects of longpass filters on the UV reaction of nucleosides. The mixed nucleoside solution without or with 1 mM SA in potassium phosphate buffer at pH 7.4 was irradiated with UV through no filter, a 300 nm longpass filter, or a 350 nm longpass filter at a temperature of 37°C for 20 min. On UV irradiation without a filter and through the 300 nm filter, addition of SA greatly enhanced the reaction of dT. However, on UV irradiation through the 350 nm filter, no reaction was observed.

Table 2 shows the effects of related compounds of SA on the reaction of nucleosides with UV light with a longer wave-



Fig. 1. (A) Time Courses of Concentration Changes in dC (Circle), dG (Square), dT (Triangle), and dA (Rhombus) When a Solution of 100 μ M Each dC, dG, dT, and dA with 1mM SA Was Irradiated with UV through a 300nm Longpass Filter in 100mM Potassium Phosphate Buffer at pH 7.4 and at a Temperature of 37°C for 0–40min; (B) The SA Dose-Dependence of Concentrations of Nucleosides When a Solution of 100 μ M dC, dG, dT, and dA with 0–2mM SA Was Irradiated with UV through a 300nm Longpass Filter in 100mM Potassium Phosphate Buffer at pH 7.4 and at a Temperature of 37°C for 20min

All the reaction mixtures were analyzed by RP-HPLC. Means \pm standard deviation (S.D.) (n = 3) are presented.

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Table 1. Effects of Longpass Filters on the Reaction of Nucleosides with UV in the Absence or Presence of SA^a)

	dC (µM)	dG (µM)	dT (µM)	dA (µM)
No filter	71.9 ± 1.5	78.5 ± 2.6	83.8 ± 0.9	95.6 ± 0.7
No filter + SA	83.4 ± 3.5	87.0 ± 0.9	6.1 ± 0.5	95.3 ± 1.3
300 nm filter	98.0 ± 2.5	97.5 ± 1.8	98.7 ± 2.3	99.1 ± 2.0
300 nm filter + SA	96.5 ± 0.8	91.4 ± 1.8	21.6 ± 1.3	98.9 ± 2.4
350 nm filter	100.5 ± 0.4	97.9 ± 0.5	99.6 ± 0.1	100.2 ± 0.1
350 nm filter + SA	101.1 ± 0.6	99.7 ± 0.8	99.1 ± 1.0	97.8 ± 0.5

a) A mixed solution of 100 µM dC, dG, dT, and dA in 100 mM potassium phosphate buffer at pH 7.4 in the absence and presence of 1 mM SA was irradiated with UV light through no filter, a 300 nm longpass filter, or a 350 nm longpass filter at a temperature of 37°C for 20 min.

Table 2. Effects of Related Compounds of SA on the Reaction of Nucleosides with UV through a 300 nm Longpass Filter^{a)}

Compounds	dC (µM)	dG (µM)	dT (µM)	dA (µM)
Salicylic acid	96.5 ± 0.8	91.4 ± 1.8	21.6 ± 1.3	98.9 ± 2.4
m-Hydroxybenzoic acid	98.3 ± 2.1	95.4 ± 2.0	94.7 ± 2.2	99.4 ± 2.0
p-Hydroxybenzoic acid	97.4 ± 0.9	99.0 ± 0.5	92.3 ± 1.1	99.6 ± 0.2
Benzoic acid	100.2 ± 1.8	99.6 ± 1.6	101.2 ± 1.4	101.2 ± 1.6
Phenol	99.5 ± 0.4	100.0 ± 0.6	99.6 ± 0.7	100.4 ± 0.6
Methyl salicylate	97.4 ± 0.3	97.7 ± 0.4	97.8 ± 0.4	99.1 ± 0.3
Acetylsalicylic acid	97.1 ± 2.9	98.1 ± 1.0	97.3 ± 0.5	99.8 ± 0.7
o-Aminobenzoic acid	98.1 ± 2.4	94.9 ± 0.3	2.1 ± 0.3	98.4 ± 0.4
<i>m</i> -Aminobenzoic acid	97.4 ± 3.0	93.3 ± 0.3	98.1 ± 2.6	98.7 ± 0.6
p-Aminobenzoic acid	92.7 ± 1.4	95.0 ± 1.7	33.8 ± 1.8	96.8 ± 1.2
Acetophenone	74.0 ± 1.0	14.8 ± 0.0	39.9 ± 2.4	79.9 ± 0.9

a) A solution of 100μ M dC, dG, dT, and dA in 100 mM potassium phosphate buffer at pH 7.4 in the absence and presence of 1 mM related compounds of SA was irradiated with UV through a 300 nm longpass filter at a temperature of 37°C for 20 min.

length than 300 nm. A solution of nucleosides $(100 \mu M \text{ each})$ with 1 mM of the compounds in 100 mM potassium phosphate buffer at pH 7.4 was irradiated with UV light through a 300 nm longpass filter at a temperature of 37°C for 20 min. The effects of acceleration on the dT reaction by *m*-hydroxybenzoic acid and *p*-hydroxybenzoic acid, which are positional isomers of salicylic acid (*o*-hydroxybenzoic acid), were rather smaller than that by salicylic acid. Benzoic acid accelerated the dT reaction, whereas *m*-aminobenzoic acid accelerated the dT reaction, whereas *m*-aminobenzoic acid did not. Acetophenone, a keton photosensitizer, accelerated the reaction of dG more than dT.

To obtain information about the reaction products from dT, a solution of 10mM dT with 1mM SA in 100mM potassium phosphate buffer at pH 7.4 was irradiated with UV through a 300nm longpass filter at a temperature of 37°C for 60min. Figure 2 shows the RP-HPLC chromatogram of the reaction mixture detected at 230nm. The UV-Vis spectra of SA and dT (Fig. 2 insets) indicated that SA can absorb UV light having a longer wavelength than 300nm, whereas dT cannot. Four major product peaks with retention times of 16.5, 17.2, 17.6, and 19.7 min, referred to as Peak 1, Peak 2, Peak 3, and Peak 4, respectively, were detected in addition to the peaks of dT and SA. All the product peaks showed similar UV spectra with λ_{max} of 215–218 nm (data not shown). The product peaks were isolated by RP-HPLC and subjected to MS and NMR. All the peaks on electrospray ionization-timeof-flight (ESI-TOF)-MS showed a signal at m/z 483 in the negative mode. High-resolution (HR)-ESI-TOF/MS values of the molecular ion for all the peaks agreed with the theoretical molecular mass for C₂₀H₂₇N₄O₁₀ within 3 ppm. For Peaks



Fig. 2. RP-HPLC Chromatogram of a Reaction Mixture of dT with SA Detected at 230 nm

The insets are on-line detected UV spectra of dT and SA. A solution of 10mM dT and 1mM SA was irradiated with UV through a 300nm lowpass filter in 100mM potassium phosphate buffer at pH 7.4 and 37°C for 60min. The HPLC system consisted of LC-10ADvp pumps and an SPD-M10Avp UV-Vis photodiode-array detector (Shimadzu, Kyoto, Japan). For the RP-HPLC, an Inertsil ODS-3 octadecylsilane column of size 4.6 ± 250 mm and a particle size of 5μ m (GL Sciences, Tokyo) was used. The eluent was 20mM ammonium acetate (pH 7.0) containing methanol. The methanol concentration was increased from 0 to 40% during 30min in linear gradient mode. The column temperature was 40°C and the flow rate was 1 mL/min.

1 and 4, ¹H-NMR showed two sets of an aliphatic proton, a methyl proton, and seven deoxyribose protons. No aromatic proton signal was observed. ¹³C-NMR showed two sets of two aliphatic carbons, two carbonyl carbons, a methyl carbon, and five deoxyribose carbons. For Peaks 2 and 3, ¹H-NMR showed a set of an aliphatic, a methyl proton, and seven deoxyribose



Fig. 3. Structures of Cyclobutane Thymidine Dimers

protons. ¹³C-NMR showed one set of two aliphatic carbons, two carbonyl carbons, a methyl carbon, and five deoxyribose carbons. Cadet et al. reported the identification of six configurationally-distinct forms of cyclobutane thymidine dimer generated from dT with UV light.5) Comparing the NMR data of the present study with the reported data, Peak 1 was identified as a single molecule of *cis-svn* thymidine dimer. In cis-syn thymidine dimer, the two nucleoside units are not symmetrically equivalent, since the symmetry is not retained by the addition of the two D-sugars (it is retained when one of two 2-deoxy-D-ribose moieties in the compound is replaced by 2-deoxy-L-ribose).⁵⁾ Peaks 2 and 3 were identified as two stereoisomers of cis-anti thymidine dimer, cis-anti (-) and cis-anti (+), respectively, in which the two nucleoside units are symmetrically equivalent. Peak 4 was identified as a single molecule of trans-anti thymidine dimer in which the two nucleoside units are not symmetrically equivalent, like Peak 1. The remaining two stereoisomers, trans-syn thymidine dimer (trans-syn (-) and trans-syn (+)), were not detected, probably because of their small production yield or overlap with other peaks. The structures of cyclobutane thymidine dimers are shown in Fig. 3.

Figure 4A shows the time-dependent changes in the concentrations of Peaks 1–4 and dT when a solution of 10 mM dT with 1 mM SA in 100 mM potassium phosphate buffer at pH 7.4 was irradiated with UV through a 300 nm longpass filter at a temperature of 37°C for up to 60 min. The concentrations of Peaks 1–4 increased with increasing irradiation time. Figure 4B shows the SA dose-dependence of concentrations of Peaks 1–4 and dT when the solution of dT with 0–2 mM SA was irradiated with UV light for 60 min. The concentration of dT decreased with increasing SA dose up to 1 mM, whereas for 1–2 mM SA, the concentrations of Peaks 1–4 and dT were unchanged. Figure 4C shows the dT dose-dependence of concentration of SA when a solution of 1 mM SA with 0–20 mM



Fig. 4. (A) The Time Course of Concentration Changes in Peak 1 (Closed Circle), Peak 2 (Closed Square), Peak 3 (Closed Triangle), and Peak 4 (Closed Rhombus) with dT (Open Triangle) When a Solution of 10mM dT with 1mM SA Was Irradiated with UV through a 300nm Longpass Filter in 100mM Potassium Phosphate Buffer at pH 7.4 and at a Temperature of 37° C for 0–60min; (B) The SA Dose-Dependence of Concentrations of Nucleosides When a Solution of 10mM dT with 0–2mM SA Was Irradiated with UV through a 300nm Longpass Filter in 100mM Potassium Phosphate Buffer at pH 7.4 and at a Temperature of 37° C for 60min; (C) The dT Dose-Dependence of Concentration of SA When a Solution of 1mM SA with 0–20mM dT in 100mM Potassium Phosphate Buffer at pH 7.4 was Irradiated with UV through a 300nm Longpass Filter at a Temperature of 37° C for 60min;

All the reaction mixtures were analyzed by RP-HPLC. Means \pm S.D. (n = 3) are presented.

dT in 100 mM potassium phosphate buffer at pH 7.4 was irradiated with UV through a 300 nm longpass filter at a temperature of 37°C for 60 min. Consumption of SA decreased with increasing dT concentration. In the presence of 10 mM dT, the consumption of SA was 0.22 mM.

Table 3 shows the effects of longpass filters on the reaction of dT with UV in the absence or presence of SA. The solution of 10mM dT without or with 1mM SA was irradiated with UV directly or through the filters at pH 7.4 and 37°C for 60 min. On UV irradiation without a filter, addition of SA enhanced the reaction of dT. On UV irradiation through the 300nm longpass filter, addition of SA greatly enhanced the reaction of dT. The total concentration of cyclobutane thymidine dimer formed was 3.18 mM. Since each thymidine dimer has two thymidine units in the structure, the concentration of total thymidine unit in products was 6.36 mM, which is almost equivalent to the consumed dT concentration. In the presence of 1 mM SA, the yields of cyclobutane thymidine dimers by UV light through 300nm longpass filter were greater than those by UV light without filter. UV-irradiation at wavelength lower than 230nm has been shown to induce efficient photoreversion, in which the cyclobutane ring of the dimers were split generating the parent dT due to direct photo absorption.²⁾ This photoreversion may explain the relatively low yields of

Table 3.	Effects of Longpass	Filters on the	Reaction of dT	with UV	in the A	Absence or Presence of SA ^a)
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	Peak 1 (mM)	Peak 2 (mM)	Peak 3 (mM)	Peak 4 (mM)	dT (mM)
No filter	0.06 ± 0.01	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	8.80 ± 0.07
No filter + SA	0.53 ± 0.01	0.13 ± 0.01	0.10 ± 0.00	0.15 ± 0.01	7.31 ± 0.03
300 nm filter	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	9.35 ± 0.03
300 nm filter + SA	1.76 ± 0.04	0.48 ± 0.01	0.29 ± 0.01	0.65 ± 0.02	3.55 ± 0.17
350 nm filter	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	9.24 ± 0.03
350 nm filter + SA	0.03 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	9.13 ± 0.02

a) A solution of 10 mM dT in 100 mM potassium phosphate buffer in the absence or presence of 1 mM SA was irradiated with UV through no filter, a 300 nm longpass filter, or a 350 nm longpass filter at pH 7.4 and at a temperature of 37°C for 60 min.

cyclobutane thymidine dimers when the irradiation was carried out without filter. On UV irradiation through the 350 nm longpass filter, almost no reaction was observed.

p-Aminobenzoic acid was widely used as an ingredient in sunscreens, since it reduced ervthema and hyperplasia of the skin by UV light.9 However, p-aminobenzoic acid has been found to photosensitize bacterial cell killing and mutation in bacterial and mammalian DNA.^{10,11)} It photosensitizes the dimerization of thymine bases in DNA upon irradiation at wavelength greater than 300 nm.¹¹⁾ However, in the International Agency for Research on Cancer (IARC) classification, p-aminobenzoic acid is categorized in Group 3 (not classifiable as to its carcinogenicity to humans), since no case reports or epidemiological studies were available.¹²⁾ SA is widely used as an ingredient in skin care products, since it can exfoliate the corneum.⁶⁾ It is reported that salicylic acid is not a photosensitizer.^{7,8)} In an animal experiment using hairless mice, salicylic acid inhibited the rate of UVB-induced nonmelanoma skin cancer.13) In the IARC classification for carcinogenicity, SA is not categorized in any group. The present study showed that the reaction of dT induced by UV (300-350nm) was greatly accelerated by SA, resulting in the formation of thymidine dimers. This implies that SA is a photosensitizer for the UV reaction of dT, resulting in cyclobutane thymidine dimers. Generally, the reaction mechanism of dT dimerization by light in the presence of photosensitizer is explained as follows; At first, ground state photosensitizer is excited to the triplet state by light irradiation. Then, the triplet energy of photosensitizer is transferred to ground state dT resulting in triplet state dT, when the triplet energy of photosensitizer is higher than or nearly equal to that of dT. The dT triplet reacts with ground state dT nearby to yield dT dimers. This reaction mechanism is proposed not only for ketone photosensitizers but also for p-aminobenzoic acid.^{2,11)} A similar reaction mechanism would be applicable to the photosensitization by SA.

The present study showed that SA is the photosensitizer for thymidine dimerization by UV light. Since SA is used as an ingredient in many cosmetics, we should pay close attention to its genotoxicity in terms of photosensitization.

Experimental

Materials Nucleosides were purchased from Sigma-Aldrich (MO, U.S.A.). Sodium salicylate and other compounds are obtained from Nacalai (Kyoto, Japan). Water was purified with a Millipore Milli-Q deionizer.

Irradiation Conditions High-intensity UV light originating from a 250W high-pressure mercury lamp (SP5-250UB, Ushio, Tokyo, Japan) with or without an optical filter through a liquid light guide was irradiated directly to the surface of a solution (1 mL) of nucleosides or dT containing sodium salicylate or other additives in 100 mM potassium phosphate buffer (pH 7.4) in a glass vial (12 mm i.d.) without a cap at 37°C. Longpass filters LU0300 (cut-on 300 nm) or LU0350 (cuton 350 nm) (Asahi Spectra, Tokyo, Japan) were used as the optical filter. The intensity of radiation on the surface of the sample solution was measured with a photometer (UIT-150, Ushio) equipped with a sensor UVD-S254 or UVD-S365. The intensities of the UV light were 57 mW/cm² for 254 nm and 487 mW/cm² for 365 nm without a filter, 1 mW/cm² for 254 nm and 490 mW/cm² for 365 nm with the 300 nm longpass filter, and 0 mW/cm² for 254 nm and 468 mW/cm² for 365 nm with the 350 nm longpass filter.

HPLC and MS Conditions The HPLC system consisted of LC-10ADvp pumps and an SPD-M10Avp UV-Vis photodiode-array detector (Shimadzu, Kyoto, Japan). For the RP-HPLC, an Inertsil ODS-3 octadecylsilane column of size $4.6 \times 250 \text{ mm}$ and a particle size of $5 \mu \text{m}$ (GL Sciences, Tokyo, Japan) was used. The eluent was 20 mM ammonium acetate (pH 7.0) containing methanol. The methanol concentration was increased from 0 to 40% during 30 min in linear gradient mode. The column temperature was 40°C and the flow rate was 1 mL/min. The RP-HPLC chromatogram was detected at 260 nm for nucleosides reactions and at 230 nm for dT reactions. ESI-TOF-MS measurements were performed on a MicroTOF spectrometer (Bruker, Bremen, Germany) in negative mode. The sample isolated by RP-HPLC was directly infused into the MS system by a syringe pump without a column.

Spectrometric Data

Peak 1 (cis-syn)

ESI-TOF-MS (negative mode): m/z 483. HR-ESI-TOF-MS (negative mode): m/z 483.173623 obsd. (Calcd for $C_{20}H_{27}N_4O_{10}$ 483.173267). UV: $\lambda_{max} = 215 \text{ nm}$ (pH 7.0). *cis-syn* A unit. ¹H-NMR (500 MHz, D_2O): δ (ppm/trimethylsilyl propanoic acid (TMSP)- d_4) 5.95 (dd, 1H, H-1'), 4.47 (ddd, 1H, H-3'), 4.40 (d. 1H. H-6), 3.92 (ddd, 1H. H-4'), 3.77 (m. 1H. H-5' or 5"), 3.74 (m, 1H, H-5' or 5"), 2.47 (m, 1H, H-2' or 2"), 2.21 (m, 1H, H-2' or 2"), 1.47 (s, 3H, CH₃). ¹³C-NMR (125 MHz, D₂O): δ (ppm/TMSP- d_4) 176.0 (C-4), 156.0 (C-2), 89.4 (C-1'), 88.5 (C-4'), 74.5 (C-3'), 65.1 (C-5'), 59.8 (C-6), 51.8 (C-5), 40.4 (C-2'), 19.9 (CH₃). *cis-syn* B unit. ¹H-NMR (500 MHz, D₂O): δ (ppm/TMSP- d_4) 5.40 (dd, 1H, H-1'), 4.42 (ddd, 1H, H-3'), 4.36 (d, 1H, H-6), 3.91 (ddd, 1H, H-4'), 3.79 (m, 1H, H-5' or 5"), 3.73 (m, 1H, H-5' or 5"), 2.58 (m, 1H, H-2' or 2"), 2.10 (m, 1H, H-2' or 2"), 1.51 (s, 3H, CH₃). ¹³C-NMR (125 MHz, D₂O): δ (ppm/TMSP-d₄) 175.2 (C-4), 155.1 (C-2), 91.6 (C-1'), 88.9 (C-4'), 73.7 (C-3'), 64.5 (C-5'), 62.7 (C-6), 49.4 (C-5), 39.2 (C-2'), 20.0 (CH₃).

Peak 2 (cis-anti (-))

ESI-TOF-MS (negative mode): m/z 483. HR-ESI-TOF/MS (negative mode): m/z 483.173473 obsd. (Calcd for $C_{20}H_{27}N_4O_{10}$ 483.173267). UV: $\lambda_{max} = 216$ nm (pH 7.0). ¹H-NMR (500 MHz, D₂O): δ (ppm/TMSP- d_4) 6.12 (dd, 1H, H-1'), 4.37 (ddd, 1H, H-3'), 4.00 (d, 1H, H-6), 3.99 (ddd, 1H, H-4'), 3.79 (m, 1H, H-5' or 5"), 3.69 (m, 1H, H-5' or 5"), 2.23 (m, 1H, H-2' or 2"), 1.94 (m, 1H, H-2' or 2"), 1.50 (s, 3H, CH₃). ¹³C-NMR (125 MHz, D₂O): δ (ppm/TMSP- d_4) 175.8 (C-4), 155.1 (C-2), 89.0 (C-4'), 87.6 (C-1'), 73.9 (CH₃).

Peak 3 (cis-anti (+))

ESI-TOF-MS (negative mode): m/z 483. HR-ESI-TOF/MS (negative mode): m/z 483.173490 obsd. (Calcd for $C_{20}H_{27}N_4O_{10}$ 483.173267). UV: $\lambda_{max} = 215$ nm (pH 7.0). ¹H-NMR (500 MHz, D₂O): δ (ppm/TMSP- d_4) 6.32 (dd, 1H, H-1'), 4.46 (ddd, 1H, H-3'), 4.16 (d, 1H, H-6), 3.96 (ddd, 1H, H-4'), 3.80 (m, 1H, H-5' or 5"), 2.32 (m, 1H, H-2' or 2"), 2.23 (m, 1H, H-2' or 2"), 1.55 (s, 3H, CH₃). ¹³C-NMR (125 MHz, D₂O): δ (ppm/TMSP- d_4) 175.4 (C-4), 155.6 (C-2), 88.3 (C-4'), 86.2 (C-1'), 74.1 (C-3'), 64.6 (C-5'), 58.2 (C-6), 52.5 (C-5), 40.2 (C-2'), 24.0 (CH₃).

Peak 4 (trans-anti)

ESI-TOF-MS (negative mode): m/z 483. HR-ESI-TOF/MS (negative mode): m/z 483.173444 obsd. (Calcd for $C_{20}H_{27}N_4O_{10}$ 483.173267). UV: $\lambda_{max} = 218 \text{ nm}$ (pH 7.0). trans-anti A unit. ¹H-NMR (500 MHz, D₂O): δ (ppm/TMSP-d₄) 6.29 (dd, 1H, H-1'), 4.36 (ddd, 1H, H-3'), 4.29 (d, 1H, H-6), 3.93 (ddd, 1H, H-4'), 3.68 (m, 1H, H-5' or 5"), 3.68 (m, 1H, H-5' or 5"), 2.39 (m, 1H, H-2' or 2"), 2.14 (m, 1H, H-2' or 2"), 1.44 (s, 3H, CH₃). ¹³C-NMR (125 MHz, D₂O): δ (ppm/TMSP-d₄) 176.9 (C-4), 155.5 (C-2), 88.3 (C-4'), 87.9 (C-1'), 74.3 (C-3'), 64.5 (C-5'), 62.2 (C-6), 49.8 (C-5), 39.3 (C-2'), 19.8 (CH₂), transanti B unit. ¹H-NMR (500 MHz, D₂O): δ (ppm/TMSP- d_4) 5.87 (dd, 1H, H-1'), 4.43 (d, 1H, H-6), 4.36 (ddd, 1H, H-3'), 3.91 (ddd, 1H, H-4'), 3.73 (m, 1H, H-5' or 5"), 3.73 (m, 1H, H-5' or 5"), 2.41 (m, 1H, H-2' or 2"), 2.18 (m, 1H, H-2' or 2"), 1.45 (s, 3H, CH₃). ¹³C-NMR (125 MHz, D₂O): δ (ppm/TMSP-d₄) 177.3 (C-4), 156.7 (C-2), 89.6 (C-1'), 88.7 (C-4'), 73.5 (C-3'), 64.5 (C-5'), 59.9 (C-6), 50.7 (C-5), 39.8 (C-2'), 20.6 (CH₂).

Quantitative Procedures For nucleosides reactions, the concentrations of the nucleosides were quantified by integrated peak areas on RP-HPLC chromatograms detected at 260 nm, and compared with those of the initial solution of nucleosides mixture. For the dT reactions, the concentrations of the prod-

ucts were evaluated according to integrated peak areas on RP-HPLC chromatograms detected at 230 nm and by the molecular extinction coefficients at 230 nm ($\varepsilon_{230 nm}$). The $\varepsilon_{230 nm}$ value of 2650 M⁻¹ cm⁻¹ was used for dT. The $\varepsilon_{230 nm}$ values of Peaks 1–4 were determined from the integration of proton signals of NMR and the HPLC peak area detected at 230 nm relative to that of dT in the mixed solution. The estimated $\varepsilon_{230 nm}$ values were 3380 M⁻¹ cm⁻¹ for Peak 1, 5130 M⁻¹ cm⁻¹ for Peak 2, 4240 M⁻¹ cm⁻¹ for Peak 3, and 5040 M⁻¹ cm⁻¹ for Peak 4.

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Conflict of Interest The authors declare no conflict of interest.

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