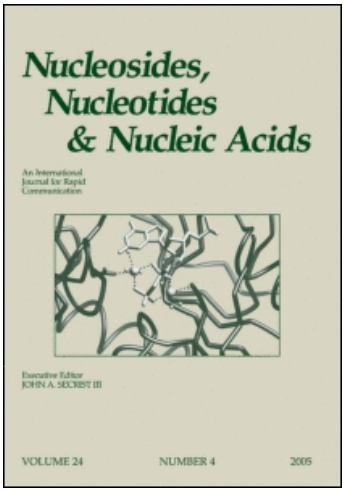

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Synthetic Nucleoside and Nucleotides. XXXIV. Photoaffinity Labeling of HIV Reverse Transcriptase: Synthesis and Utilization of 2',3'-Dideoxy Uridylate Analogs Bearing Aryl(trifluor0methyl)-Diazirine Moiety

Toyofumi Yamaguchi[a]; Mineo Saneyoshi[a]
[a] Department of Biological Sciences, The Nishi-Tokyo University, Yamanashi, Japan

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SYNTHETIC NUCLEOSIDE AND NUCLEOTIDES. XXXIV. PHOTOAFFINITY LABELING OF HIV REVERSE TRANSCRIPTASE: SYNTHESIS AND UTILIZATION OF 2',3'-DIDEOXY URIDYLATE ANALOGS BEARING ARYL(TRIFLUOROMETHYL)-DIAZIRINE MOIETY †,[1]

Toyofumi Yamaguchi * and Mineo Saneyoshi

Department of Biological Sciences, The Nishi-Tokyo University, 2525 Yatsuzawa, Uenohara-machi, Kitatsuru-gun, Yamanashi 409-01, Japan

ABSTRACT: In order to develop a photoaffinity labeling reagent for HIV-1 reverse transcriptase, a 2',3'-dideoxyUTP analog bearing a photo-reactive group at the 5-position of the uracil ring, 2',3'-dideoxy-*E*-5-[4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]styryl]-UTP, was designed and synthesized. This photo-reactive analog could preferentially bind to the 66 kDa subunit of the p66/p51 heterodimeric HIV-1 reverse transcriptase under irradiation by near-ultraviolet light (365 nm).

The nucleoside analogs such as 3'-azido-3'-deoxythymidine (AZT), 2',3'-dideoxycytidine (DDC), 2',3'-dideoxyinosine (DDI) and 2',3'-didehydro-3'-deoxythymidine (D4T) inhibit the replication of human immunodeficiency viruses type 1 (HIV-1) and have been used clinically in the treatment of acquired immunodeficiency syndrome (AIDS). These nucleosides are known to be phosphorylated and exert their activity by inhibiting HIV reverse transcriptase [2-6]. Thus, HIV reverse transcriptase has been proved to be one of the important target molecules for developing anti-HIV agents. The 5'-triphosphates of the 2',3'-dideoxythymidine analogs including AZT 5'-triphosphate (AZT-TP), 2',3'-dideoxythymidine 5'-triphosphate (ddTTP) and D4T 5'-triphosphate have been demonstrated to be potent inhibitors of HIV-1 reverse transcriptase [7-9]. Therefore, it is important to investigate the three-dimensional interactions between these 2',3'-dideoxythymidine 5'-triphosphate analogs and the amino acid residues at the active site of the HIV reverse transcriptase molecule in comparison with eukaryotic DNA polymerases. We have attempted to

† This paper is dedicated to Dr. Yoshihisa Mizuno on occasion of his 75th birthday.

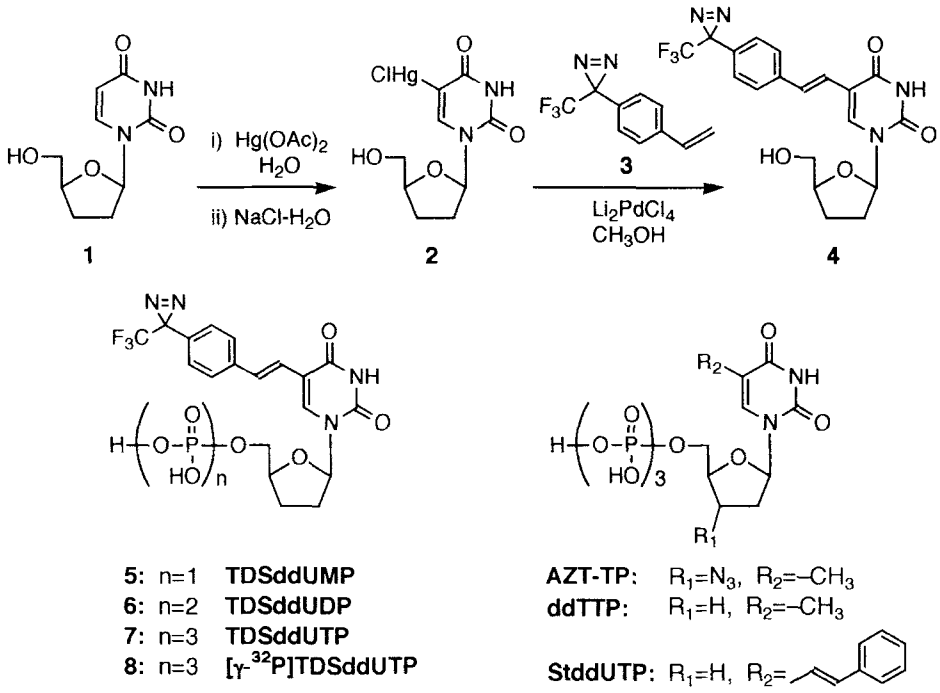

FIG. 1. Reaction scheme for the synthesis of 2',3'-dideoxy-*E*-5-[4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]styryl]uridine (**4**) and the structures of phosphorylated derivatives of **4** , AZT-TP, ddTTP and StddUTP.

find a photoaffinity labeling reagent for the active site(s) of HIV-1 reverse transcriptase and examine the labeling of this enzyme. From the results of crystallographic analysis [10,11] and our labeling experiment of HIV-1 reverse transcriptase, structural information about the catalytic site(s) of HIV-1 reverse transcriptase will be obtained, and these may be useful in designing a selective inhibitor for the enzyme. In a previous study, we demonstrated that the inhibitory effect of 2',3'-dideoxy-*E*-5-styryluridine 5'-triphosphate (StddUTP), which bears steric and hydrophobic styryl groups at the 5-position of the uracil ring on the HIV-1 reverse transcriptase, was as potent as ddTTP [12]. Using this information, we have designed 2',3'-dideoxy-*E*-5-[4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]styryl]UTP (TDS-ddUTP) (**7**) and its radiolabeled form **8** which have a photoreactive aryl(trifluoromethyl)-diazirine group as the carbene precursor. This diazirine group is stable and easily handled

for labeling studies compared to the aryl azido functional group [13,14]. The present paper deals with the synthesis and application of TDSddUTPs (**7** and **8**).

MATERIALS AND METHODS

UV spectra were recorded on a Beckman DU70 recording spectrophotometer. ^{1}H-NMR spectra were obtained on a JEOL GSX400 NMR spectrometer with tetramethylsilane as an internal standard. Mass spectra were measured on a JEOL JMS-01 SG-2 spectrometer. High-performance liquid chromatography (HPLC) was performed using a Shimadzu LC-9A apparatus, with a flow rate of 0.80 ml/min. *Method A*—A YMC Pack ODS A-302 (YMC Co., Ltd.) reversed phase column (4.5 mm x 15 cm) was used. The solvent was aqueous CH_3CN containing 0.05 M Et_3NHOAc (pH 7.0). *Method B*—A TSK-GEL DEAE-2SW (Tohso Co., Ltd.) ion exchange column (4.5 mm x 25 cm)was used, with 0.21 M potassium phosphate buffer (pH 6.95) containing 20% (v/v) CH_3CN as the solvent. The analyses were performed at 45 °C.

[^{3}H]dTTP and [γ-^{32}P]ATP were purchased from Amersham. Yeast nucleoside 5'-diphosphate kinase was purchased from Sigma. Recombinant HIV-1 reverse transcriptase, which showed 51 and 66 kDa bands in approximately equimolar proportions on SDS-PAGE, was purchased from Seikagaku Kogyo.

2',3'-Dideoxy-*E*-5-[4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]styryl]-uridine (4). A solution of mercuric acetate (7.0 g, 22 mmol) in water (40 ml) was added to a mixture of 2',3'-dideoxyuridine (**1**) (4.5 g, 21.2 mmol) dissolved in water (30 ml), and the mixture was stirred for 8 h at 50 °C. After the addition of NaCl (3 g, 52 mmol), the mixture was stirred for 1 h at room temperature. The precipitates were collected by filtration and washed with 0.15 M aqueous sodium chloride (200 ml), ethanol (30 ml) and finally with ether (30 ml). Crude 5-chloromercuri-2',3'-dideoxyuridine (**2**) (5.5 g, 13 mmol) was obtained as a white solid and used in the next step without further purification. The well dried **2** (1.77 g, 4.0 mmol) was suspended in methanol (25 ml), and then 3-(4-ethenylphenyl)-3-trifluoromethyl-3*H*-diazirine (**3**)[15] (1.02 g, 4.8 mmol) and a methanolic solution of 0.1 M lithium tetrachloropalladate (42 ml) were added. After being refluxed for 5 h, the reaction mixture was filtered and the filtrate was evaporated. The residue was chromatographed on a column of silica gel (100 g) with dichloromethane–ethylacetate–methanol (20:5:1, v/v). Between the two major products, the early-eluting fractions were collected and evaporated to dryness. The residue was

chromatographed again under the same conditions. The fractions containing nucleoside **4** were combined and the solvent was removed under reduced pressure to give **4** as yellowish solid. 0.17 g (0.4 mmol, 10%). HPLC retention time: 3.99 min (*Method A*, 60% CH_3CN–0.05M Et_3NHOAc). UV (H_2O) λmax 316 nm (ε 22200), (50 mM NaOH) λmax 321 nm (ε 23700). ^{1}H-NMR (DMSO-d_6) δ: 1.90 (m, 2 H, H-3'), 2.05 (m, 1 H, H-2'), 2.30 (m, 1 H, H-2'), 3.60 (m, 1 H, H-5'), 3.80 (m, 1 H, H-5'), 4.07 (m, 1 H, H-4'), 5.26 (dd, 1 H, 5'-OH), 5.97 (dd, 1 H, H-1'), 6.98 (d, 1 H, olefinic-H, J = 15 Hz), 7.22 (d, 2 H, aromatic-H), 7.41 (d, 1 H, olefinic-H, J = 15 Hz), 7.57 (d, 1 H, aromatic-H), 8.44 (s, 1 H, H-6). HR-MS *m/z*: 422.1236 and 394.1173 (M^+–N_2). Calcd. for $C_{19}H_{17}F_3N_4O_4$: 422.1202 and 394.1140.

2',3'-Dideoxy-*E*-5-[4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]styryl]-uridine 5'-monophosphate (5). Phosphoryl chloride (0.2 ml, 2.14 mmol) was added dropwise to a solution of the nucleoside **4** (85 mg, 0.21 mmol) in triethyl phosphate (2 ml) at –10 °C, and the solution was stored for 1 day at 4 °C. The mixture was poured into 0.1 M sodium bicarbonate (100 ml) with stirring and extracted with chloroform (10 ml x 2). The aqueous layer was applied to a column of DEAE-cellulose (2.5 cm x 20 cm, bicarbonate form) pre-equilibrated with water. Elution was performed with a linear gradient (2,000 ml) of 0 to 0.5 M triethylammonium bicarbonate (pH 7.8). The main peak of UV absorption, eluted at 0.30 to 0.35 M, was collected and evaporated to dryness. In order to remove residual triethylammonium bicarbonate, the resulting residue was co-evaporated with water. The 5'-monophosphate **5** (3,400 OD_{315} units, 0.16 mmol) was isolated as the triethylammonium salt in 73% yield.

2',3'-Dideoxy-*E*-5-[4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]styryl]-uridine 5'-triphosphate (7) and 5'-diphosphate (6). A mixture of 5'-monophosphate **5** (388 OD_{315} units, 0.018 mmol) and 1,1'-carbonyldiimidazole (14.4 mg, 0.088 mmol) in dimethylformamide (DMF) (1 ml) was stirred for 2.5 h at room temperature, and methanol (2.7 μl, 0.067 mmol) was added to the mixture. After stirring for 30 min, a solution of tri-*n*-butylammonium pyrophosphate (0.6 M, 0.3 ml) in DMF was added and the mixture was stirred for 24 h at room temperature. The solvent was removed under reduced pressure, and the residual syrup was dissolved in water (50 ml). Active carbon (Norit A) (0.4 g) was added to the solution and the mixture was stirred for 15 min. The mixture was filtered and the active carbon was washed well with water. Elution of the nucleotides from the active carbon was performed with 1.5 M ammonium

hydroxide containing 50% (v/v) ethanol. The eluate was evaporated to dryness, and the residue was dissolved in water (50 ml). The solution was applied to a column of DEAE-cellulose (2.5 cm x 10 cm, bicarbonate form) pre-equilibrated with water. The column was washed with 0.1 M triethylammonium bicarbonate (pH 7.8), and the elution was performed with a linear gradient (600 ml) of 0.10 to 0.70 M triethylammonium bicarbonate (pH 7.8). The main peak of UV absorption, which eluted at 0.5 to 0.6 M, was collected and evaporated to dryness. After co-evaporation with water, the 5'-triphosphate (**7**) (158 OD_{315} units) was isolated as the triethylammonium salt with a 40% yield. About 10 OD_{315} units of the product was further purified by HPLC (*Method A*, 27% (v/v) CH_3CN–0.05 M Et_3NHOAc). Finally, **7** was analyzed by HPLC (*Method A*, 30% (v/v) CH_3CN–0.05 M Et_3NHOAc) and the purity of **7** assessed by measuring UV absorption at 315 nm was confirmed to be greater than 95%.

In a similar fashion, the 5'-diphosphate (**6**) was synthesized, using the reaction of tri-*n*-butylammonium phosphate instead of tri-*n*-butylammonium pyrophosphate. HPLC retention times of 5'-di- and 5'-triphosphates (**6** and **7**) using *Method B* were 11.4 and 14.3 min respectively. 5'-Dephosphorylation of the di- and triphosphate (**6** and **7**) was carried out with bacterial alkaline phosphatase (Nippon Gene, *E. coli* C75) using 20 nmol of **6** or **7** and 1 unit of the enzyme in 0.1 M Tris–HCl (pH 8.0), 1 mM $MgCl_2$ in a total volume of 250 μl. Incubation was performed for 2 h at 37 °C. The digested products were confirmed by comparison with the nucleoside (**4**) using HPLC (*Method A*, 60% (v/v) CH_3CN–0.05 M Et_3NHOAc).

[γ-^{32}P]2',3'-Dideoxy-*E*-5-[4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]-styryl]UTP (8). Nucleoside 5'-diphosphate kinase (2.5 units) in 0.1 M Tris-HCl (pH 7.5) (12 μl) was added to an aqueous solution (18 μl) of 2',3'-dideoxy-*E*-5-[4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]styryl]UDP (**6**) (0.5 OD_{315} units, 23 nmol) and [γ-^{32}P]ATP (3.0 MBq, 0.12 nmol), and the mixture was incubated for 1 h at 37 °C. The product was chromatographed on a DEAE-cellulose column (0.8 cm x 9 cm) with a linear gradient (100 ml) of 0 to 1.0 M triethylammonium bicarbonate (pH 7.8). The main peak of radioactivity was collected and evaporated to dryness. The final product (2.0 MBq) was stored at –20 °C in the dark.

Assay of HIV-1 reverse transcriptase activity. The reaction mixture (25 μl) comprised 50 mM Tris-HCl (pH 8.3), 1 mM dithiothreitol, 100 μg/ml bovine serum albumin, 50 mM KCl, 0.5 mM $MnCl_2$, 60 μg/ml poly (A)-oligo$(dT)_{12-18}$ (2:1, w/w), 50

μM [^{3}H]dTTP (12 Bq/pmol), various concentrations of inhibitors and 5 ng HIV-1 reverse transcriptase. Incubation was carried out for 20 min at 37 °C. The reaction mixtures were then chilled and transferred to DE81 ion-exchange papers (Whatman). The papers were washed with 5% Na_2HPO_4 (six times) and dried. The radioactivity of the polynucleotides retained on the paper was then measured. When kinetic analysis was performed, the concentrations of [^{3}H]dTTP and the inhibitors were varied.

Photoaffinity labeling of HIV-1 reverse transcriptase. The reaction mixture (7.5 μl) comprised 50 mM Tris-HCl (pH 8.3), 1 mM dithiothreitol, 50 mM KCl, 80 μg/ml poly(A), 20 μg/ml oligo(dT)$_{12\text{-}18}$, 5.6 kBq [γ-^{32}P]TDSddUTP (**8**), 40 ng HIV-1 reverse transcriptase and 0 or 0.5 mM $MnCl_2$. When the effect of dTTP was examined, 10 μM dTTP was also included. Incubation was carried out for 5 min at 25 °C in the dark. After chilling the reaction mixture, it was irradiated with a 100W black-light lamp (UVP, Inc. BA100AF) at a distance of 8 cm for 15 min on an ice bath. The mixture was mixed with 7.5 μl of the solution consisting of 0.1 M Tris-HCl (pH 6.8), 4% SDS, 20% (v/v) glycerol, 50 mM dithiothreitol and 0.02% bromophenol blue, and then heat-denatured for 5 min at 95 °C. The samples were then subjected to SDS-PAGE. After detection of the protein bands by staining with Coomassie brilliant blue, the gel was dried and exposed to X-ray film (Konica) with an intensifying screen for 2 to 6 h at –70 °C. The radioactivity associated with the 66 kDa polypeptide was determined by Cerenkov counting after excising the radioactive band from gel.

SDS-PAGE. SDS-PAGE was carried out according to the method of Laemmli [16], using 10% separation gel and 5% condensation gel. Protein bands were detected by staining with Coomassie brilliant blue.

RESULTS

Synthesis. For the synthesis of an uracil nucleoside bearing a styryl group at the 5-position of the uracil ring, a method using an organopalladium intermediate was utilized[17,18]. In the present study, 2',3'-dideoxyuridine was converted to its 5-mercurated derivative **2** by treatment with mercuric acetate and sodium chloride [19], and then 3-(4-ethenylphenyl)-3-trifluoromethyl-3*H*-diazirine (**3**)[15] was reacted with **2** in the presence of lithium tetrachloropalladate in methanol. After purification by silica gel column chromatography, the desired nucleoside **4** was obtained as a yellowish powder. Proton NMR spectra of this nucleoside **4** showed that the coupling constant (J) between the vinylic

protons of the styryl moiety at the 5-position was 15 Hz, indicating that the styryl substituent was "trans" [17]. Compound **4** was then converted to the corresponding 5'-monophosphate **5** by phosphorylation with phosphoryl chloride in triethyl phosphate [20], and further phosphorylated to the 5'-diphosphate **6** and 5'-triphosphate **7** by the phosphoroimidazolidate method [21]. The isotope-labeled compound **8** was prepared from the corresponding 5'-diphosphate **7** and [γ-^{32}P]ATP, catalyzed by nucleoside diphosphate kinase essentially as described by Glynn and Chappell [22].

Inhibitory effect of 2',3'-dideoxy-*E*-5-[4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]styryl]UTP (TDSddUTP, 7) on HIV-1 reverse transcriptase in the dark. We measured the inhibitory effect of TDSddUTP (**7**) on HIV-1 reverse transcriptase with poly(A)-oligo(dT) as the template-primer comparing it with ddTTP and StddUTP. As shown in Figure 2A, HIV-1 reverse transcriptase activity was inhibited strongly by these three compounds, and 50% inhibition of TDSddUTP (**7**) was observed at about 0.5 μM in the presence of 50 μM dTTP. From double reciprocal plots, the mode of inhibition of HIV-1 reverse transcriptase by TDSddUTP (**7**) was shown to be competitive with respect to dTTP (Figure 2B). The *K*i value of TDSddUTP (**7**) for HIV-1 reverse transcriptase was determined as 0.075 μM (Table 1), and was about 180 times smaller than the *K*m of dTTP (14 μM).

Photoaffinity labeling of HIV-1 reverse transcriptase. The radioactive analog **8** was treated with HIV-1 reverse transcriptase and irradiated with near UV light (365 nm) at 0 °C. The radiolabeled product was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by autoradiography. The effects of Mn^{2+} and dTTP on the photoaffinity labeling reaction were examined using poly(A)-oligo(dT) as the template-primer. As shown in Figure 3, radioactivity was detected at Mr = 66,000 in the absence of Mn^{2+}, and generation of the radiolabeled polypeptide was inhibited by addition of 10 μM dTTP. Under the condition of photolabeling, approximately 3% of [γ-^{32}P]TDS-ddUTP (**8**) was converted into the covalent complex which was detected at Mr = 66,000. No loss of HIV-1 reverse transcriptase activity was detected during the above irradiation procedure in the absence of TDSddUTP (**7**).

DISCUSSION

For developing a photoaffinity labeling reagent for HIV reverse transcriptase and other DNA polymerases which are sensitive to 2',3'-dideoxynucleoside 5'-triphosphates, we

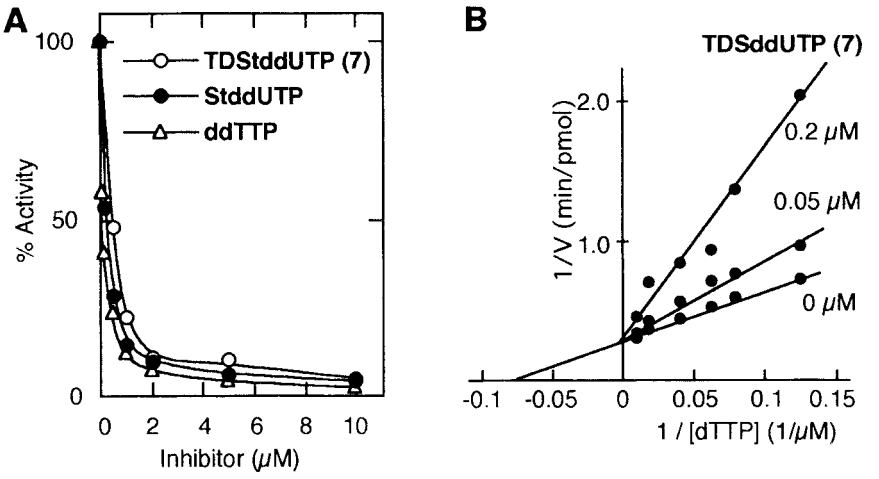

FIG. 2. **A.** Inhibitory effects of TDSddUTP (**7**), StddUTP and ddTTP on HIV-1 reverse transcriptase in the dark. Inhibitory effects of these compounds were measured with poly(A)-oligo(dT) as the template-primer in the presence of 50 μM [^{3}H]dTTP. **B.** Lineweaver-Burk plot for the inhibition of HIV-1 reverse transcriptase by TDSddUTP (**7**) in the dark. Poly(A)-oligo(dT) was used as the template-primer.

TABLE 1. Kinetic constants of HIV-1 reverse transcriptase for TDSddUTP (**7**), StddUTP and ddTTP. Poly(A)-oligo(dT) was used as the template-primer.

	Ki (μM)	Mode of inhibition
Inhibition by		
TDSddUTP (**7**)	0.075	competitive [c]
StddUTP	0.050	competitive [c]
ddTTP	0.040	competitive [c]
Km for [^{3}H]dTTP	14	

[c] Competitive with respect to dTTP.

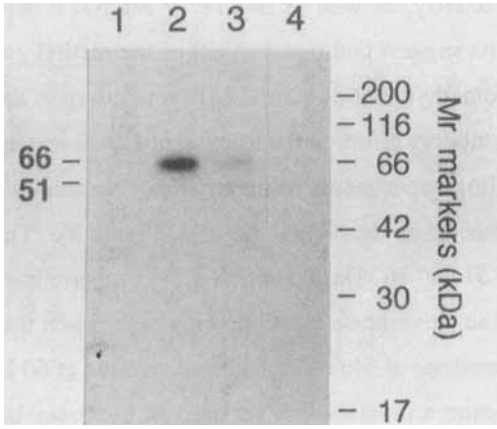

FIG. 3. Photoaddition of [γ-^{32}P]TDSddUTP (**8**) to HIV-1 reverse transcriptase. A mixture was incubated for 5 min at 25 °C in the dark using poly(A)-oligo(dT) as the template-primer, and subjected to near UV irradiation. The radiolabeled product was analyzed by SDS-PAGE, and detected by autoradiography as described in "MATERIALS AND METHODS". Lane **1**, with 0.5 mM $MnCl_2$; **2**, without $MnCl_2$; **3**, without $MnCl_2$, but with 10 μM dTTP; **4**, without $MnCl_2$, and no photolysis control.

designed and synthesized a 2',3'-dideoxyUTP analog (**7** and **8**) bearing an aryl(trifluoromethyl)diazirine system which was proved to be suitable for photo-reactive cross-linking[13,14]. In this study, 2',3'-dideoxyuridine (**1**) was converted to its mercurated derivative **2**, and then reacted with a styrene analog **3** in the presence of lithium tetrachloropalladate in methanol. The desired nucleotide **7** and its [γ-^{32}P] labeled form (**8**) were synthesized by chemical and enzymatic phosphorylation of the nucleoside **4**. As a photoaffinity labeling reagent for HIV-1 reverse transcriptase, tritium-labeled BI-RJ-70, an arylazido photoaffinity analog of Nevirapine, has been synthesized and successfully used for labeling HIV-1 reverse transcriptase. However, this compound binds to a noncatalytic modulatory site of the enzyme [23,24]. As labeling reagents which could bind to the dNTP binding site of DNA polymerases by a photo-reaction, the nucleotide analogs bearing an arylazido group, 8-azido-dATPfor *E. coli* DNA polymerase I [25] and *E*-5-(4-azidostyryl)araUTP for cherry salmon DNA polymerase α [26], were reported.

In a previous paper, we reported that 2',3'-dideoxy-*E*-5-styrylUTP showed a remarkable inhibitory effect on HIV-1 reverse transcriptase in a competitive fashion with respect to the substrate dTTP [12]. In this study, we demonstrated that TDSddUTP (**7**) also

inhibited the enzyme activity, as well as ddTTP or StddUTP, with the same mode of inhibition. These results suggest that replacement of the methyl group of ddTTP with a styryl or 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]styryl group at the 5-position does not particularly affect the inhibitory effect on the reaction of HIV-1 reverse transcriptase.

Photoaffinity labeling experiments using a radioactive analog **8** showed that radiolabeled product was detected as the band at 66 kDa (Figure 3). This result is consistent with the fact that of the 51 and 66 kDa subunits of HIV-1 reverse transcriptase, only the 66 kDa subunit catalyses the polymerase reaction. However, when the labeling experiment was performed in the presence of Mn^{2+}, the radioactive band at 66 kDa was not detected. Mn^{2+} is one of the common divalent cations used in the assay of DNA polymerases, providing the Mn–dNTP chelates. Incorporation of the dNMP moiety and release of pyrophosphate occurs by nucleophilic attack of the 3'-hydroxyl group at the 3' terminus of the primer on the α phosphate of the Mn–dNTP chelate [9]. ^{32}P of labeling reagent **8** may well have been eliminated from the photoaffinity labeling reagent as pyrophosphate before the photo-reaction in our experiments. We found that the photolabeling of HIV-1 reverse transcriptase with cold TDSddUTP (**7**) and poly(A)-[^{32}P]oligo(dT)$_{16}$ as the template-primer in the presence of Mn^{2+} gave a radioactive polypeptide at about 80 kDa (data not shown). From this preliminary data, it seems likely that the photolabile primer analog was formed by addition of TDSddUMP onto [^{32}P]oligo(dT)$_{16}$ and then could covalently bind to the enzyme molecule. As shown in Figure 3, generation of the radiolabeled product was strongly inhibited by addition of dTTP, indicating that TDSddUTP (**8**) could bind to the dTTP binding site of HIV-1 reverse transcriptase.

We have already reported in a previous study that the *K*i value of StddUTP for DNA polymerase γ was 10 times smaller than that for HIV-1 reverse transcriptase when poly(A)-oligo(dT) was used as the template-primer [12]. In the present study, we suggest that our newly synthesized labeling reagents **7** and **8** should be useful tools for elucidation of the active site, and particularly the dTTP binding site, of eukaryotic DNA polymerase γ as well as HIV-1 reverse transcriptase.

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