Deoxyadenosine-Based DNA Polymerase Photoprobes: Design, Synthesis, and Characterization as Inhibitors of the *Escherichia coli* DNA Polymerase I Klenow Fragment[†,‡]

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ABSTRACT: DNA polymerase photoprobes 2-[(4-azidophenacyl)thio]-2′-deoxyadenosine 5′-triphosphate (**1**), 2-[(4-azidophenylsulfenyl)thio]-2′-deoxyadenosine 5′-triphosphate (**2**), and 2-[(4-azido-2-nitrophenyl)-thio]-2′-deoxyadenosine 5′-triphosphate (**3**) were designed from a thermodynamic model of DNA polymerase I−substrate interactions such that the triphosphate would anchor the inhibitor and allow the phenyl azide to interact with the complementary template binding site. Photoprobes **1**−**3** were synthesized by condensation of 2-thio-2′-deoxyadenosine or its phosphate with *p*-azidophenacyl bromide, *N*-(4-azidophenylsulfenyl)phthalimide, and 4-azido-1-fluoro-2-nitrobenzene, respectively, and characterized as reversible and photoinduced irreversible inhibitors of the DNA polymerase I Klenow fragment and HIV I reverse transcriptase. The aryl azides decomposed with irradiation at 300 and 350 nm with half-lives ranging from 0.98 to 2.33 min and 2.15 to 5.38 min, respectively, with quantum efficiencies ranging from 0.29 to 0.55 and no apparent photodecomposition of the 2-thio-2′-deoxyadenosine nucleotide. Photoprobes **1**−**3** showed mixed noncompetitive inhibition of the Klenow fragment polymerase activity *versus* poly(dA)·$(T)_{10}$ as variable substrate with apparent competitive inhibition constants of 2.1, 36, and 29 μM, respectively, evidence suggesting that these photoprobes bind to both the free enzyme form and the enzyme−template-primer binary complex. Of the three photoprobes, only nucleotide **1** photoinactivates the Klenow fragment; in the presence of a 200-fold excess of nitrene scavenger, photoprobe **1** inactivates 92% of the Klenow fragment polymerase activity with saturation observed at 9.7 μM and an IC_{50} of about 2 μM. This evidence demonstrates that photoprobe **1** does bind to the Klenow fragment in the absence of template-primer and that it is an efficient photoprobe.

Substrate recognition by DNA polymerases and the structure of polymerase−DNA−deoxynucleotide triphosphate (dNTP)[1] ternary complexes have been the recent focus of extensive studies attempting to elucidate fundamental mechanisms of DNA polymerization and repair. In addition, models of the active ternary complexes may generate information useful for the design of specific inhibitors of DNA replication. One approach to the identification of molecular contacts between polymerases and their substrates is nucleotide photoaffinity labeling. Examples of deoxynucleotide photoprobes available for this task include 8- and 2-azido-2′-deoxyadenosine phosphates (Evans & Coleman, 1989; Flaherty et al., 1995; Rush & Konigsberg, 1990) and more recently photoprobes based on TTP (Cheng et al., 1993; Satav et al., 1990) and its analogs, particularly 5-iodo- (Willis et al., 1993) and 5-azido-dUTP (Evans & Haley, 1987).

Our approach to the design of extended deoxynucleotide-based photoaffinity labels uses the thermodynamic model for substrate interactions in DNA polymerase I (Doronin et al., 1989; Kolocheva et al., 1991; Lavrik et al., 1987; Potapova et al., 1990; Yadav et al., 1992). In the model, template-primer binding is stabilized by multiple interactions, including the 3′-hydroxyl and phosphate anion of the primer (−5.8 kcal/mol), phosphoryl oxygens and phosphate anions of the template (−5.9 kcal/mol), and hydrophobic interactions of the bases (−0.32 kcal mol^{-1} $base^{-1}$). In contrast, dNTP binding in the ternary complex is dominated by Mg^{2+}-coordinated triphosphate binding interaction (−6.7 kcal/mol), with a small contribution of −0.35 kcal/mol in base pairing interaction with the complementary template. The predominant importance of the triphosphate of the dNTPs in binding to DNA polymerase makes it a good anchor point for investigating the topology of the enzyme surrounding this binding site. Thus, both 8-azido-dATP (Rush & Konigsberg, 1990) and TTP (Pandey et al., 1987) are photolabels of the DNA polymerase I Klenow fragment. The disadvantage of these types of photoprobes is that the affinity of the polymerase for dNTPs decreases by more than 10-fold without template-primer, presumably due in part to the loss in binding interaction associated with base pairing to the complementary template (McClure & Jovin, 1975; Muise & Holler, 1985). For example, the IC_{50} for photoincorporation of 8-azido-dATP into the Klenow fragment is 35 μM, a value close to the K_d for dATP binding to the free enzyme form but >10-fold higher than the K_m for binding of dATP

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[1] Abbreviations: TEAB, triethylammonium bicarbonate; HPLC, high-pressure liquid chromatography; FAB^+MS, fast atom bombardment mass spectroscopy; dNTP, 2′-deoxynucleotide 5′-triphosphate; pol α, polymerase α.

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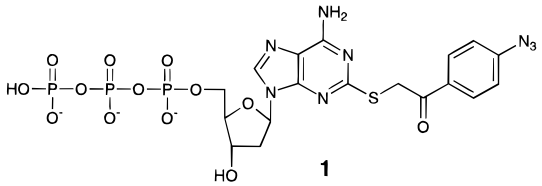
to the enzyme−template-primer binary complex (Rush & Konigsberg, 1990), and although the apparent IC_{50} for photoinactivation of the Klenow fragment by TTP was not determined, only 20% photoinactivation was observed at 10 μM (Pandey et al., 1987).

We hypothesized that a hydrophobic aryl azide tethered to the minor groove side of a purine nucleotide base would provide an extension that would interact with the complementary template binding region on the enzyme. In this way, the photolabel would have increased binding interaction through hydrophobic contact and bring an aryl azide into this binding region. Examples of this approach are seen in the extensive work of Wright and Brown (Wright & Brown, 1990). The dGTP analog N^2-(4-*n*-butylphenyl)-2′-deoxyguanosine 5′-triphosphate is a potent inhibitor of DNA polymerase α (pol α) with a K_i of about 10 nM (Khan et al., 1984). Additionally, the structurally related analog of dATP, i.e., N^2-(4-*n*-butylphenyl)-dATP, is similarly active against pol α (Khan et al., 1985). Thus, the minor groove substituent in these polymerase inhibitors is an important determinant of binding to pol α, although none of these polymerase inhibitors is active against prokaryotic enzymes such as DNA polymerase I (Khan et al., 1984, 1985; Wright & Brown, 1990). We felt that increasing the tether link between the nucleotide base and the phenyl ring, or an aryl azide for photoprobes, might increase the potential for Klenow fragment interaction at sites extended out from the dNTP binding site. Thus, we designed photoprobes **1**−**3** where the nucleotide base is separated from the aryl azide by three-, two-, and one-atom tethers, respectively. We chose adenosine as



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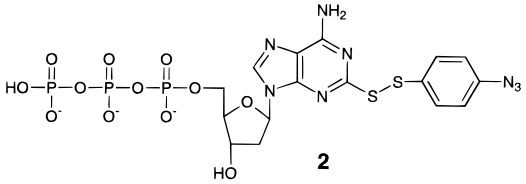

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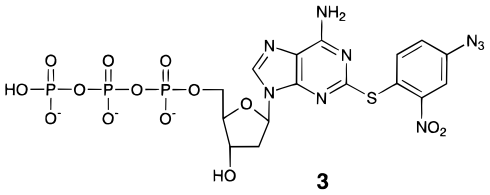

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the nucleotide base because it is the most hydrophobic (Knorre et al., 1988) and the least photoactive (Satav et al., 1990) of the four natural bases. In this paper, we report on the synthetic, photochemical, and enzyme inhibitory activities of these probes, and in the following paper, we report on the characterization of photoprobe **1** as an affinity label of the DNA polymerase I Klenow fragment (Moore et al., 1996).

EXPERIMENTAL PROCEDURES

Materials and Methods. All chemicals obtained commercially were of reagent or analytical grade. Analytical thin layer chromatography was performed on 0.25 cm silica plates obtained from Sigma (St. Louis, MO). Flash chromatography was carried out with Selectro Scientific silica gel particle size 32−63 obtained from Fischer (Pittsburgh, PA). Ion exchange chromatography was conducted with DEAE Sephadex resin (Pharmacia, Piscataway, NJ). High-pressure liquid chromatography was performed on a Beckman gradient system using System Gold software. Reverse phase separations were conducted with Vydac C_{18} analytical and semipreparative columns using the following methods: method A, analytical column with a 30 min gradient elution from 20 mM triethylammonium bicarbonate (TEAB) to 20 mM TEAB in 50% acetonitrile at a flow rate of 1.0 mL/min; method B, semipreparative column with a 50 min gradient elution from 20 mM TEAB to 20 mM TEAB in 50% acetonitrile at 2.5 mL/min; and method C, the same as method A except ammonium acetate buffer (10 mM, pH 6.8) was substituted for TEAB. In all cases, elution was monitored by UV absorbance at 90% (longer wavelength side) of the λ_{max} for the compound under analysis.

Corrected melting points were determined in a Thomas Hoover capillary melting point apparatus. The ^{1}H and ^{31}P NMR spectra were recorded on a Varian XL 300 spectrometer, and the chemical shifts (in parts per million) are reported relative to the solvent peaks in DMSO-d_6, $CDCl_3$, and D_2O for ^{1}H and phosphoric acid for ^{31}P. The IR spectra were recorded on a Bomem Michaelson 120 FT-IR instrument, and the ultraviolet spectra were recorded on a Hewlett-Packard 8450A diode array spectrophotometer. Mass spectral data for electron ionization and chemical ionization were obtained on a Nermag (Paris, France) R10-10 quadruple GC/MS system. Positive and negative fast atom bombardment mass spectra (FAB^+MS) were obtained on a VG Analytical ZAB spectrophotometer. Photolysis was conducted in a Rayonet Photochemical Mini-Reactor chamber Model RMR-600 purchased from the Southern New England Ultraviolet Co. (Branford, CT). Liquid scintillation was conducted in a Packard 1900 TR Liquid Scintillation Counter (Meridian, CT).

The methodology of Beaman and Robins (Beaman & Robins, 1962) was used to prepare 2,6-dichloropurine from 2,6-purinedithiol. Nucleoside **4** (2-thio-2′-deoxyadenosine) was prepared by the sodium salt glycosylation method (Kazimierczuk et al., 1984; Revankar & Robins, 1989). The methodology of Vanin et al. (Vanin & Tae, 1981) was used to prepare *N*-(4-azidophenylsulfenyl)phthalimide. In general, the nucleotides were too hygroscopic for combustion analysis; however, all products were >99% pure on the basis of analytical thin layer chromatography, reverse phase HPLC, and ^{1}H and ^{31}P NMR spectroscopy.

*Synthesis of 2-[(2-Cyanoethyl)thio]-2′-deoxyadenosine (**5**).* Nucleoside **4** (401 mg, 1.4 mmol) was dissolved in 0.68 N NaOH (2.5 mL), and the solution was treated with acetonitrile (2.5 mL) containing 3-bromopropionitrile (0.38 g, 2.80 mmol). After the mixture was stirred for 18 h, flash silica (1 g) was added and the mixture was dried. The dried material was purified by flash chromatography, eluting with CH_2Cl_2/MeOH (9:1). The glassy solid was quickly dissolved in 1 mL of CH_2Cl_2 and diluted with 10 mL of MeOH. Crystallization of **5** occurred after 1 h at ambient temperature. The crystals were collected by centrifugation and dried under high vacuum, yielding 323 mg (68%) of pure **5** as white crystals: mp 158−159 °C; R_f 0.51 (8:2 CH_2Cl_2/MeOH); ^{1}H

NMR (300 MHz, DMSO-d_6) δ 8.24 (s, 1H, C_8H), 7.47 (bs, 2H, NH_2), 6.28 (t, J = 6.84 Hz, 1H, $C_1'H$), 5.32 (d, J = 4.20 Hz, 1H, $C_3'OH$), 4.95 (t, J = 5.64 Hz, 1H, $C_5'OH$), 4.38 (m, 1H, $C_3'H$), 3.84 (dd, J = 2.91, 4.62 Hz, 1H, $C_4'H$), 3.55 (m, 2H, $C_5'H$), 3.32 (t, J = 6.69 Hz, 2H, CH_2CN), 3.00 (t, J = 6.57 Hz, 2H, CH_2S), 2.71 (m, 1H, $C_2'H$), 2.27 (m, 1H, $C_2'H$); UV−vis (0.1 N NaOH) λ_{max} 230 (21 300), 275 (13 400); high-resolution FAB$^+$MS *m/e* 337.1113 [calcd for $C_{13}H_{17}N_6O_3S$ (M + 1): 337.1083].

*Synthesis of 2-[(2-Cyanoethyl)thio]-2′-deoxyadenosine 5′-Monophosphate (**6**).* Nucleoside **5** (300 mg, 0.89 mmol) in freshly distilled triethyl phosphate (6 mL) was cooled to −10 °C (methanol/ice bath) and treated with freshly distilled phosphorus oxychloride (205 mg, 1.20 mmol). After the mixture was stirred for 2 h at −10 °C, 8 mL of saturated $NaHCO_3$ was added, followed by 8 mL of chloroform. The layers were separated, and the chloroform layer was extracted twice with water (10 mL). The aqueous layer was diluted to 200 mL with water, loaded on a 1.5 × 20 cm DEAE column, and eluted with a gradient of 300 to 700 mM triethylammonium bicarbonate (200 mL each). Two peaks were collected and lyophilized, yielding 67.1 mg (22%) of starting material and 202 mg (55%) of **6**: mp 192 °C turns brown, does not melt below 300 °C; R_f 0.53 (20:12:1 propanol/NH_4OH/H_2O); ^{1}H NMR (300 MHz, D_2O) δ 8.21 (s, 1H, C_8H), 6.38 (t, J = 6.84 Hz, 1H, $C_1'H$), 4.66 (m, 1H, $C_3'H$), 4.20 (m, 1H, $C_4'H$), 3.97 (t, J = 4.41 Hz, 2H, $C_5'H$), 3.35 (t, J = 6.45 Hz, 2H, CH_2CN), 2.98 (t, J = 6.36 Hz, 2H, SCH_2), 2.77 (m, 1H, $C_2'H$), 2.53 (m, 1H, $C_2'H$); ^{31}P NMR (300 MHz, D_2O) δ 0.57; UV−vis (0.1 N NaOH) λ_{max} 231 (17 300), 274 (11 700); high-resolution FAB$^+$MS *m/e* 415.0592 [calcd for $C_{13}H_{16}N_6O_6PS$ (M − 1) 415.0590].

*Synthesis of 2-Thio-2′-deoxyadenosine 5′-Monophosphate (**7**).* Nucleotide **6** (147 mg, 0.35 mmol) in concentrated NH_4-OH (50 mL) was treated at 60 °C in a sealed tube equipped with a stirring bar. After 6 h, the solution was cooled, excess ammonia was removed under vacuum, and the solution was lyophilized, yielding 122 mg (95%) of **7**: mp >300 °C; R_f 0.13 (20:12:1 propanol/NH_4OH/H_2O); ^{1}H NMR (300 MHz, D_2O) δ 8.28 (s, 1H, C_8H), 6.30 (t, J = 6.36 Hz, 1H, $C_1'H$), 4.63 (m, 1H, $C_3'H$), 4.19 (m, 1H, $C_4'H$), 3.94 (t, J = 3.81 Hz, 2H, $C_5'H$), 2.64 (m, 1H, $C_2'H$), 2.52 (m, 1H, $C_2'H$); ^{31}P NMR (300 MHz, D_2O) δ 0.51; UV−vis (0.1 N NaOH) λ_{max} 242, 284; high-resolution FAB$^+$MS *m/e* 362.0312 [calcd for $C_{10}H_{13}N_5O_6PS$ (M − 1) 362.0324].

*Synthesis of 2-[(4-Azido-2-nitrophenyl)thio]-2′-deoxyadenosine (**8**).* Nucleoside **4** (99.6 mg, 0.35 mmol), DBU (58.5 mg, 0.35 mmol), and 4-azido-1-fluoro-2-nitrobenzene (71.2 mg, 0.39 mmol) were dissolved in acetonitrile/water (2:1, 1.5 mL) and stirred for 6 h at room temperature. Flash silica (1 g) was added after 6 h, the solvent was removed, and the mixture was resolved by flash chromatography by eluting with methylene chloride/methanol (9:1), yielding 138.7 mg (88%) of **8**: R_f 0.26 (9:1 CH_2Cl_2/MeOH); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.18 (s, 1H, C_8H), 7.81 (d, J = 8.52 Hz, 1H, 3-ArH), 7.70 (d, J = 2.52 Hz, 1H, 5-ArH), 7.39 (dd, J = 2.49, 8.58 Hz, 1H, 6-ArH), 6.22 (t, J = 6.63 Hz, 1H, $C_1'H$), 4.32 (m, 1H, $C_3'H$), 3.94 (m, 1H, $C_4'H$), 3.58 (t, J = 3.87 Hz, 2H, $C_5'H$), 2.74 (m, 1H, $C_2'H$), 2.30 (m, 1H, $C_2'H$); IR (KBr) 2112, 2129 (N_3), 1530, 1335 cm^{-1} (NO_2); high-resolution FAB$^+$MS *m/e* 446.0979 [calcd for $C_{16}H_{16}N_9O_5S$ (M + 1) 446.0995].

*Synthesis of 2-[(4-Azidophenacyl)thio]-2′-deoxyadenosine 5′-Monophosphate (**9**).* Nucleotide **7** (48.5 mg, 0.11 mmol) was treated with 4′-azido-2-bromoacetophenone (26.4 mg, 0.12 mmol) and triethylamine (41 mg, 0.24 mmol) in DMF for 20 min. The solvent was removed under high vacuum, and the residue was suspended in water and converted to the sodium salt by titration with 1.0 M NaOH. Insoluble material was removed by centrifugation, and the product was purified from the supernatant by HPLC using method B, yielding 52.6 mg (75%) of **9**: R_f 0.53 (20:12:1 propanol/NH_4OH/H_2O); ^{1}H NMR (300 MHz, D_2O) δ 8.18 (s, 1H, C_8H), 8.05 (d, J = 8.70 Hz, 2H, *o*-COArH), 7.16 (d, J = 8.67 Hz, 2H, *m*-COArH), 5.66 (t, J = 6.84 Hz, 1H, $C_1'H$), 4.38 (m, 1H, $C_3'H$), 3.92 (m, 1H, $C_4'H$), 3.82 (m, 2H, $C_5'H$), 2.51 (m, 1H, $C_2'H$), 2.06 (m, 1H, $C_2'H$); ^{31}P NMR (300 MHz, D_2O) δ −0.28; IR (KBr) 2128, 2107 (N_3), 1647 (carbonyl), 1320 (purine NH_2), 1290 (P═O), 1053 cm^{-1} (POH); UV−vis (0.1 N NaOH) λ_{max} 227 (32 181), 278 (30 145); high-resolution FAB$^+$MS *m/e* 521.0757 [calcd for $C_{18}H_{18}N_8O_7SP$ (M − 1) 521.0757].

*Synthesis of 2-[(4-Azidophenylsulfenyl)thio]-2′-deoxyadenosine 5′-Monophosphate (**10**).* Nucleotide **10** was prepared from **7** (40 mg, 0.11 mmol) and *N*-(4-azidophenylsulfenyl)phthalimide (32.9 mg, 0.11 mmol) using the method described above for the synthesis of **9**. The product was purified by HPLC using method C. Repeated lyophilizations yielded 12 mg (21%) of **10**: R_f 0.48 (20:12:1 propanol/NH_4OH/H_2O); ^{1}H NMR (300 MHz, D_2O) δ 8.19 (s, 1H, C_8H), 7.38 (d, J = 7.80 Hz, 2H, *o*-SArH), 6.75 (d, J = 7.68 Hz, 2H, *m*-SArH), 6.29 (t, J = 5.46 Hz, 1H, $C_1'H$), 4.55 (m, 1H, $C_3'H$), 4.14 (m, 1H, $C_4'H$), 3.96 (m, 2H, $C_5'H$), 2.66 (m, 1H, $C_2'H$), 2.37 (m, 1H, $C_2'H$); ^{31}P NMR (300 MHz, D_2O) δ −0.25; IR (KBr) 2132, 2097 (N_3), 1350 (purine NH_2), 1301 (P═O), 1050 cm^{-1} (POH); UV−vis (0.1 N NaOH) λ_{max} 222 (26 941), 269 (27 476); high-resolution FAB$^+$MS *m/e* 511.0348 [calcd for $C_{16}H_{16}N_8O_6S_2P$ (M − 1) 511.0372].

*Synthesis of 2-[(4-Azido-2-nitrophenyl)thio]-2′-deoxyadenosine 5′-Monophosphate (**11**).* Nucleoside **8** (7.3 mg, 16 mM) was phosphorylated by the same procedure used for phosphorylation of **6**. The product was purified by HPLC method B on a semipreparative scale, yielding 2.6 mg (31%) of **11** and 2.9 mg (40%) of starting material: R_f 0.59 (20:12:1 propanol/NH_4OH/H_2O); ^{1}H NMR (300 MHz, D_2O) δ 8.22 (s, 1H, C_8H), 7.79 (d, J = 2.46 Hz, 1H, 3-ArH), 7.70 (d, J = 8.43 Hz, 1H, 5-ArH), 7.35 (dd, J = 2.52, 8.52 Hz, 1H, 6-ArH), 6.22 (t, J = 6.84 Hz, 1H, $C_1'H$), 4.36 (m, 1H, $C_3'H$), 4.11 (m, 1H, $C_4'H$), 3.84 (t, J = 5.28 Hz, 2H, $C_5'H$), 2.67 (m, 1H, $C_2'H$), 2.39 (m, 1H, $C_2'H$); ^{31}P NMR (300 MHz, D_2O) δ 0.16; IR (KBr) 2130, 2112 (N_3), 1530 (NO_2), 1355 (purine NH_2), 1305 (P═O), 1065 cm^{-1} (POH); UV−vis (0.1 N NaOH) λ_{max} 258 (21 620), 268 (21 650); high-resolution FAB$^+$MS *m/e* 524.0518 [calcd for $C_{16}H_{14}N_9O_8SP$ (M − 1) 524.0502].

*Synthesis of 2-[(4-Azidophenacyl)thio]-2′-deoxyadenosine 5′-Triphosphate (**1**).* Nucleotide **9** (4.9 mg, 9.5 μmol) and *n*-tributylamine (2.5 mg, 9.5 μmol) in DMF (250 μL) treated with carbonyldiimidazole (7.8 mg, 48 μM) in DMF (250 μL). After 30 min of stirring at ambient temperature, the mixture was stored in a desiccator for 20 h. The excess carbonyldiimidazole was destroyed by the addition of 3.1 μL of methanol. The reaction was allowed to continue for 30 min, and the tributylamine salt of pyrophosphate (21.8 mg, 48

μmol) in DMF (200 μL) was added and stirred for 30 min. The mixture was stored in a desiccator for 8 h, the solvent was removed (high vacuum at 50 °C), and the mixture was purified by HPLC method B, yielding 1.5 mg (23% by UV) of **1**: R_f 0.11 (20:12:1 propanol/NH_4OH/H_2O); ^{1}H NMR (300 MHz, D_2O) δ 8.21 (s, 1H, C_8H), 8.14 (d, J = 8.76 Hz, 2H, *o*-COArH), 7.25 (d, J = 8.67 Hz, 2H, *m*-COArH), 5.62 (t, J = 6.72 Hz, 1H, $C_1'H$), 4.51 (m, 1H, $C_3'H$), 4.09 (m, 1H, $C_4'H$), 3.93 (m, 2H, $C_5'H$), 2.53 (m, 1H, $C_2'H$), 2.02 (m, 1H, $C_2'H$); ^{31}P NMR (500 MHz, D_2O) δ −7.25 (d, J = 48.5 Hz, γP), −8.24 (d, J = 48.7 Hz, αP), −19.99 (t, J = 48.3 Hz, βP); UV−vis (0.1 N NaOH) λ_{max} 227, 278 (peak ratio of 1.068:1); high-resolution FAB$^+$MS *m/e* 681.0094 [calcd for $C_{18}H_{20}N_8O_{13}SP_3$ (M − 1) 681.0083].

Synthesis of 2-[(4-Azidophenylsulfenyl)thio]-2′-deoxyadenosine 5′-Triphosphate (2). Nucleotide **10** (10.4 mg, 20.3 μmol) was triphosphorylated as described above for the synthesis of **1** and was purified by HPLC method C, yielding 1.2 mg (9% by UV) of **2**: R_f 0.13 (20:12:1 propanol/NH_4OH/H_2O); ^{1}H NMR (300 MHz, D_2O) δ 8.23 (s, 1H, C_8H), 7.42 (d, J = 8.43 Hz, 2H, *o*-SArH), 6.78 (d, J = 8.43 Hz, 2H, *m*-SArH), 6.31 (t, J = 6.93 Hz, 1H, $C_1'H$), 4.57 (m, 1H, $C_3'H$), 4.14 (m, 1H, $C_4'H$), 3.94 (t, J = 4.89 Hz, 2H, $C_5'H$), 2.66 (m, 1H, $C_2'H$), 2.38 (m, 1H, $C_2'H$); UV−vis (0.1 N NaOH) 221, 269 (peak ratio of 0.98:1); high-resolution FAB$^+$MS *m/e* 670.9694 [calcd for $C_{16}H_{18}N_8O_{12}S_2P_3$ (M − 1) 670.9699].

2-[(4-Azido-2-nitrophenyl)thio]-2′-deoxyadenosine 5′-Triphosphate (3). Nucleotide **11** (5.6 mg, 10.6 μM) was triphosphorylated as described above for the synthesis of **1**, yielding 972 μg (13% by UV) of **3** [1.12 mg (23%) of starting material was recovered]: R_f 0.09 (20:12:1 propanol/NH_4OH/H_2O); ^{1}H NMR (300 MHz, D_2O) δ 8.28 (s, 1H, C_8H), 7.82 (d, J = 2.52 Hz, 1H, 3-ArH), 7.71 (d, J = 8.64 Hz, 1H, 5-ArH), 7.37 (dd, J = 2.52, 8.60 Hz, 1H, 6-ArH), 6.23 (t, J = 6.45 Hz, 1H, $C_1'H$), 4.50 (m, 1H, $C_3'H$), 4.15 (m, 1H, $C_4'H$), 4.01 (dd, J = 4.74, 4.98 Hz, 2H, $C_5'H$), 2.98 (m, 1H, $C_2'H$), 2.38 (m, 1H, $C_2'H$); ^{31}P NMR (300 MHz, D_2O) δ −4.43 (γP), −5.02 (αP), −9.79 (βP); UV−vis (0.1 N NaOH) λ_{max} 222, 269 (peak ratio of 1:1); high-resolution FAB$^+$MS *m/e* 683.9825 [calcd for $C_{16}H_{17}N_9O_{14}SP_3$ (M − 1) 683.9829].

Nucleotide Photochemistry. Photolysis of compounds **9**−**11** was conducted in a Rayonet photochemical reactor (eight medium-pressure lamps) in a water-jacketed quartz cell. The cell was positioned at the center of the reactor (4.5 cm from the source), and the lamp output was standardized using the chemical actonometry method (Hatchard & Parker, 1956). Aqueous solutions of **9** (2.40 mM), **10** (7.05 mM), and **11** (2.15 mM) were photolyzed, and samples were removed at various time intervals (∼0.15 s to 2 min) for UV analysis. The decrease in absorbance of the aryl azide chromophore as a function of time was fit to eq 1,

$$\frac{A_t - A_\infty}{A_o - A_\infty} = \exp(-kt) \qquad (1)$$

where A_∞ is the absorbance at infinite time of photolysis, A_t is the absorbance at time t, A_o is the initial absorbance, and k is the rate constant for the reaction. Quantum efficiencies were calculated from the first-order rate constant for photodecomposition as a function of light flux using eq 2,

$$\Phi = \frac{[P](V)(0.5)}{(\text{lamp output})(0.693/k)} \qquad (2)$$

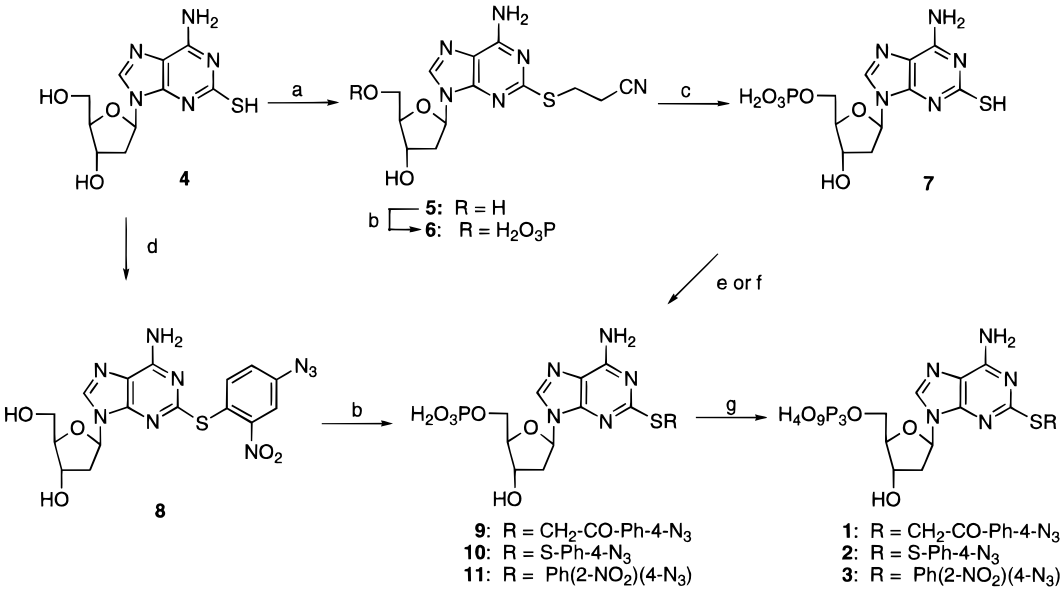
where [P] is the molar concentration of the photoprobe, V is the volume of the photolyzed solution in liters, lamp output is in h, k is the rate constant for photolysis in h^{-1}, and Φ is the quantum efficiency of the process.

Steady State Kinetic Inhibition Assays. DTT was removed from the stock enzyme by dialysis against 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM β-mercaptoethanol and 50% glycerol at 10 °C using Spectra/Por dialysis tubing (molecular weight cutoff of 10 000). The same procedure was used to remove β-mercaptoethanol for assays involving photoprobe **2**, except β-mercaptoethanol was absent from the buffer. Protein concentrations following dialysis were determined using the microanalysis protocol for the BCA protein assay in microtiter plates *versus* BSA as a standard.

The standard assay buffer contained 50 mM Tris-HCl buffer (pH 7.5) containing 2 mM $MgCl_2$; substrate mixtures in assay buffer contained the following: for variable TTP, five concentrations of [^{3}H]TTP (25.5 cpm/pmol) from 8 to 80 μM containing 8 nM poly(dA)·$(T)_{10}$; and for variable template-primer, five concentrations of poly(dA)·$(T)_{10}$ from 10 to 200 nM containing 3.5 or 200 μM [^{3}H]TTP. These substrate solutions and two units of enzyme containing three concentrations of inhibitor (0 to 10 μM for **1** and 0 to 50 μM for **2** and **3**) in assay buffer were equilibrated separately at 37 °C for 5 min, and the reaction was initiated by addition of enzyme to substrate. The reaction was quenched at two time intervals from 0 to 30 min by addition of aliquots to an equal volume of 300 mM EDTA. We limited these experiments to two time points since minimizing the total time for each experiment (i.e., variable substrate *versus* inhibitor) improved reproducibility, and control experiments demonstrated that the initial velocity was linear over the 30 min time interval. The quenched samples were subsequently spotted in triplicate on 2 × 2 cm^2 swatches of DEAE ion exchange paper. The papers were washed three times in 300 mM ammonium formate (pH 8.0) with gentle stirring. The papers were dehydrated in 95% ethanol followed by an ether wash, each for 5 min (Brutlag & Kornberg, 1972). Papers were air-dried and transferred into 7 mL liquid scintillation vials containing 5 mL of Scintiverse II, and each square was counted for 5 min. The initial velocity was determined as the picomoles of [^{3}H]TTP incorporated into DNA per minute, and the data in the absence and presence of inhibitor were analyzed for competitive, noncompetitive, and uncompetitive inhibition using the steady state kinetic equations for inhibition of an enzyme with a sequential-ordered kinetic mechanism (Cleland, 1963a−c), where $k_{is,app}$ is the apparent inhibition constant at the free enzyme and $k_{ii,app}$ is the apparent inhibition constant at the binary enzyme−DNA complex.

Photoinactivation of the Klenow Fragment. A standard photolysis mixture contained 66.7 mM Tris-HCl buffer (pH 7.5), containing 2.67 mM $MgCl_2$, 2 mM β-mercaptoethanol, 0.1 μM Klenow fragment (dialyzed to remove DTT), and varying concentrations of photoprobes **1**−**3**. Mixtures were pre-equilibrated for 5 min at 37 °C and then transferred to a water-jacketed quartz photolysis cell equilibrated at 37 °C.

Scheme 1[a]



[a] (a) CH_3CN, DBU, 3-bromopropionitrile; (b) $POCl_3$, $(EtO)_3PO$; (c) NH_4OH; (d) 4-azido-1-fluoro-2-nitrobenzene, Bu_3N; (e) 4′-azido-2-bromoacetopheone, Bu_3N; (f) *N*-(4-azidophenylsulfenyl)phthalimide; (g) (i) DMF, CDl, (ii) MeOH; (iii) $(Bu_3N)_{1.5}H_4O_7P_2$, DMF.

Each sample was photolyzed at 3000 Å and 37 °C for 5 min in a Rayonet photochemical reactor (eight medium-pressure lamps). Aliquots of the mixture were removed at time 0 and 5 min, diluted 1:60 with Tris-HCl assay buffer, and stored on ice. The samples were assayed for residual polymerase activity using the general assay protocol described above using 200 μM [^{3}H]TTP and 200 nM poly(dA)•$(T)_{10}$. The activity after 5 min of photolysis is given as a percentage of activity before photolysis.

RESULTS

Photoprobe Synthesis and Photochemistry. DNA polymerase nucleotide photoprobe synthesis entailed the introduction of an aryl azide into 2-thio-2′-deoxyadenosine 5′-monophosphate (Scheme 1). However, conversion of **4** to the O_5' monophosphate **7** by traditional methods (Yoshikawa et al., 1969) resulted in alkylation of the 2-thio group by the triethyl and trimethyl phosphate. An alternative methodology using the selective 5′-hydroxyl phosphorylating reagent bis(1,1,1-trichloroethyl)phosphochloridate (Franke et al., 1968; Gerrard et al., 1954) was successful. However, deprotection could not be effected because the zinc employed formed salts with the thio group, preventing the recovery of product. We subsequently observed that protection of the 2-thio group of **4** with a cyanoethyl group (Hurd & Gershbein, 1947) allowed for synthesis of the protected 5′-monophosphate. Deprotection of **6** in aqueous ammonia generated the target compound 2-thio-2′-deoxyadenosine 5′-monophosphate (**7**).

The side chains for the 4-azidophenacyl and 4-azidophenylsulfenyl derivatives of **7** were introduced in DMF with triethylamine using 4′-azido-2-bromoacetophenone and *N*-(4-azidophenylsulfenyl)phthalimide, respectively. However, the 4-azido-2-nitrophenyl group could not be introduced by reaction of **7** with 4-azido-1-fluoro-2-nitrobenzene; apparently, 4-azido-1-fluoro-2-nitrobenzene (a hard electrophile) reacts with the phosphate anion (a hard nucleophile) rather than with the free thiol (soft nucleophile), thus generating an activated phosphate ester which hydrolyzes during workup. Nevertheless, it was found that 2-thio-2′-deoxyadenosine (**4**) reacts with 4-azido-1-fluoro-2-nitrobenzene in aqueous acetonitrile; therefore, nucleoside **8** was prepared first, followed by phosphorylation to the monophosphate **11** (the other aryl azides did not survive these acidic phosphorylation conditions). The site of aryl azide addition was confirmed by the disappearance of the thiolactam peaks (1414 and 1214 cm^{-1}) in the IR spectra of the products. The monophosphates were subsequently converted to the triphosphates using the carbonyldiimidazole activation technique (Hoard & Ott, 1987).

Studies into purification schemes for the mono- and triphosphates resulted in a novel purification methodology. Initially, ion exchange chromatography by column and FPLC techniques was attempted in the purification, but triphosphate analogs could not be eluted even under high salt conditions (2 M ammonium acetate); also, purification by reverse phase HPLC using either 0.1% TFA or 10 mM ammonium acetate resulted in poor recovery yields. Nevertheless, triethylammonium bicarbonate has been used for the purification of nucleotides by column chromatography (Hata et al., 1975; Hoard & Ott, 1987; Waters & Connolly, 1992; Zimmet et al., 1993), and in addition, tri- and tetrabutylammonium salts have been used as ion-pairing reagents in nucleotide separations using analytical HPLC techniques (Ingebresten & Bakken, 1982; Juengling & Kemmermeier, 1980; Perrone & Brown, 1984; Walseth et al., 1980). The similarities in these buffers prompted us to examine the triethylammonium bicarbonate system in reverse phase chromatography since Goody and Isakov (1986) successfully employed the acetate salt in HPLC purification of nucleotides. This system had the advantage of ion pairing with the ability to lyophilize the buffer (the tri- and tetrabutylammonium salts cannot be removed by lyophilization), and TEAB provided a good NMR probe for determining the efficiency of salt removal. The triethylammonium bicarbonate system was efficient at mono-, di-, and triphosphate separation, providing samples of high purity as determined by TLC, analytical HPLC, and ^{1}H and ^{31}P NMR spectroscopy. Unfortunately, this buffer system was not applicable to preparative purifications of **2**

Table 1: Photodecomposition Half-Lives and Quantum Efficiencies of Nucleotide Photoprobe Monophosphates

compound	photolysis wavelength (nm)	$t_{1/2}$ (min)	quantum efficiency (Φ)
9	300	0.98	0.43
9	350	2.15	0.39
10	300	1.34	0.29
10	350	2.65	0.28
11	300	2.33	0.55
11	350	5.38	0.46

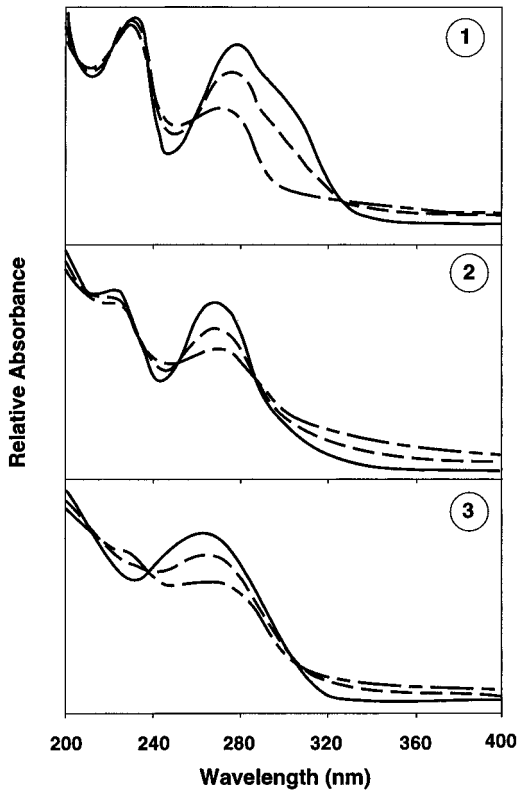


FIGURE 1: UV spectra of **9** (top), **10** (middle), and **11** (bottom) before photolysis (top spectrum in each panel) and after photolysis for one half-life (middle spectrum in each panel) and seven half-lives (bottom spectrum in each panel). Nucleotides were photolyzed at 300 nm as described in Experimental Procedures.

and **10**. The basic pH of TEAB promoted facile disulfide exchange; therefore, an ammonium acetate buffer at low pH was used for preparative purification of these nucleotides.

The rate and efficiency of photodecomposition of compounds **9**−**11** were determined to assess their compatibility with biomolecules and their mode and rate of decomposition. It was preferred that the kinetics of photodecomposition ($t_{1/2}$) and quantum efficiency (Φ) be short and high, respectively. Photodecomposition of **9** at 300 and 350 nm was followed by the decrease in absorbance of the phenacyl azide at 298 nm; likewise, decomposition of **10** and **11** was monitored at 266 and 262 nm, respectively. The half-lives ($t_{1/2}$) and quantum efficiencies (Φ) are presented in Table 1, and sample UV spectra from the photodecomposition studies are given in Figure 1. In general, the UV spectra showed isobestic points through at least seven half-lives, and the $t_{1/2}$ values ranged from 0.98 to 2.3 min at 300 nm and from 2.2 to 5.3 min at 350 nm, with the 4-azido-2-nitrophenyl analog having the longest $t_{1/2}$. Additionally, the quantum efficiencies for the photochemical process ranged from 0.28 to 0.58, with the 4-azidophenylsulfenyl analog **10** being the least efficient.

Table 2: Klenow Fragment Steady State Inhibition Patterns and Constants for Photoprobes **1**−**3**

photo-probe	variable substrate	inhibition type	$K_{is,app}$ (μM)	$K_{ii,app}$ (μM)
1	TTP[a]	competitive	−	4.0
1	poly(dA)·$(T)_{10}$[b]	uncompetitive	−	5.2
1	poly(dA)·$(T)_{10}$[c]	mixed noncompetitive	2.1	4.3
2	TTP[a]	mixed noncompetitive	110	18
2	poly(dA)·$(T)_{10}$[b]	mixed noncompetitive	36	8.8
3	TTP[a]	competitive	−	15
3	poly(dA)·$(T)_{10}$[b]	mixed noncompetitive	29	4.4

[a] Poly(dA)·$(T)_{10}$ was maintained at 8 nM. [b] TTP was maintained at 200 μM. [c] TTP was maintained at 3.3 μM.

Photoprobe Enzyme Inhibition Activity. Photoaffinity probes **1**−**3** were screened as inhibitors of the polymerase activity of the Klenow fragment using a poly(dA)·$(T)_{10}$ and [^{3}H]TTP substrate system, a template-primer system noncomplementary to the photoprobes. Saturation (data not shown) of dTTP and poly(dA)·$(T)_{10}$ was achieved with Michaelis constants of 6.7 μM and 24 nM, respectively, kinetic constants comparable to literature values (Bryant et al., 1983; Carroll & Benkovic, 1990; Mizrahi et al., 1985).

The initial velocity, steady state kinetics for reversible inhibition of the Klenow fragment in the dark was investigated as a function of both variable TTP and poly(dA)·$(T)_{10}$ concentration, and results of these experiments are summarized in Table 2. Photoprobes **1**−**3** were competitive, mixed noncompetitive, and competitive inhibitors, respectively, of the Klenow fragment with TTP as the variable substrate [at a nonsaturating concentration of poly(dA)·$(T)_{10}$], with $K_{ii,app}$ values of 4.0, 18, and 15 μM, respectively, and a $K_{is,app}$ of 110 μM for photoprobe **2**. Interestingly, when poly(dA)·$(T)_{10}$ was the variable substrate and TTP was held constant at saturation, photoprobe **1** showed uncompetitive inhibition with a $K_{ii,app}$ of 5.2 μM, which reverted to mixed noncompetitive inhibition at subsaturating concentrations of TTP with a $K_{is,app}$ of 2.1 μM and a $K_{ii,app}$ of 4.3 μM. In contrast, photoprobes **2** and **3** were mixed noncompetitive inhibitors of the Klenow fragment *versus* varying poly(dA)·$(T)_{10}$ at saturating TTP with $K_{is,app}$ values of 36 and 29 μM and $K_{ii,app}$ values of 8.8 and 4.4 μM, respectively. In contrast, photoprobe **1** was inactive as an inhibitor of the HIV I reverse transcriptase at concentrations up to 100 μM.

Nucleotides **1**−**3** were investigated as photoprobes by photolysis of a 0.1 μM solution of Klenow fragment at 300 nm in 67 mM Tris-HCl buffer (pH 7.5) containing 2.67 mM $MgCl_2$, 2.0 mM β-mercaptoethanol as a scavenger, and variable concentrations of photoprobe for 5 min; the results of these experiments are recorded in Table 3. Photolysis of the Klenow fragment for 5 min in the absence of **1** caused a 21% decrease in polymerase activity. In contrast, photolysis of the Klenow fragment with 0.97, 1.7, 4.8, and 9.7 μM **1** for 5 min at 300 nm led to 13, 47, 90, and 92% inactivation relative to control. Thus, the photoinactivation data show saturation at 9.7 μM with nearly complete inactivation. In addition, when the Klenow fragment was equilibrated with 9.7 μM **1** for 5 min in the dark and then assayed for activity, the enzyme retained 91% of the initial activity, results demonstrating the light dependency of inactivation. In contrast to the concentration-dependent photoinactivation of the Klenow fragment by **1**, photoprobe **3** failed to photoinactivate the Klenow fragment above

Table 3: Photoinactivation of the Klenow Fragment by Adenosine-Based Photoprobes

photoprobe	conditions[a]	[photoprobe] (μM)	% remaining activity[b]
−	photolysis	0	79
1	photolysis	0.97	87
1	photolysis	1.7	53
1	photolysis	4.8	10
1	photolysis	9.7	9
1	no photolysis	9.7	91
3	photolysis	60	86
3	photolysis	400	56

[a] Photolysis was carried out using a 0.1 μM solution of the Klenow fragment in 67 mM Tris-HCl buffer (pH 7.5) containing 2.67 mM $MgCl_2$ and 2 mM β-mercaptoethanol. Photolysis was carried out in a quartz cell at 300 nm for 5 min. [b] Remaining activity relative to a control assay determined at time 0 before photolysis.

control at 60 μM and showed only partial photoinactivation at 400 μM.

DISCUSSION

The aryl azides incorporated into photoprobes **1**−**3** have been previously utilized as photoaffinity reagents. However, with the exception of the 4-azidophenacyl group which was incorporated into 5-thio-UTP (Hanna et al., 1989) and 5-thio-CTP (Hanna et al., 1993), these aryl azides have been incorporated into compounds distinctly different from nucleoside triphosphates (Fleet et al., 1969; Hagedorn et al., 1978; Vanin & Tae, 1981). The unique structural properties of **1**−**3** necessitated evaluation of their photochemical properties since wavelengths shorter than 300 nm rapidly damage biomolecules and prolonged irradiation even at longer wavelengths can result in decomposition of enzymes and other proteins (Bayley & Knowles, 1977; Williams & Konigsberg, 1991). In all three cases, the UV spectra as a function of photolysis time displayed isosbestic points through seven half-lives, evidence for a single reaction pathway, with the drop in UV absorbance corresponding to the absorption maxima of the aryl azide. Thus, the UV sensitive portion of these nucleotides is the aryl azide and not the 2-thio-2′-deoxyadenosine, results consistent with the observation that adenosine nucleotides are very weakly UV active in photolabeling studies even at low-wavelength photolysis (Satav et al., 1990). Additionally, the half-life and quantum efficiency of photodecomposition of each aryl azide corresponds to that observed for similar chromophores in other systems. Thus, the nucleotide and tether length has no apparent chemical effect on the photochemical properties of these aryl azides.

The sequential-ordered kinetic mechanism for DNA polymerization catalyzed by the Klenow fragment has been extensively characterized by Benkovic and co-workers (Carroll & Benkovic, 1990; Kuchta et al., 1988; Mizrahi et al., 1985, 1986; Weisman-Shomer et al., 1989). Initiation of polymerization requires ordered binding of template-primer to the free enzyme form of the Klenow fragment, followed by addition of a complementary base-paired dNTP to the enzyme−template-primer binary complex. This sequence is followed by a conformational change that generates a catalytically competent ternary complex. The competitive inhibition of the Klenow fragment observed for photoprobes **1** and **3** as a function of variable TTP requires a mutually exclusive interaction with the same enzyme form, i.e., the binary enzyme−template-primer complex. In contrast, noncompetitive inhibition for a multisubstrate enzyme suggests that the inhibitor binds to a form different from the variable substrate and that the two forms are not separated by an irreversible step (Cleland, 1963a−c). However, processivity of the DNA polymerase complicates interpretation of kinetic data analyzed by the Michaelis−Menten formulation since substrate complexes may not be in rapid equilibrium between the free and bound states, although even in this case, mixed noncompetitive inhibition relates to the interaction of an inhibitor with multiple enzyme forms (Cho et al., 1994). Thus, one interpretation of the mixed noncompetitive inhibition patterns observed for **3** *versus* TTP as the variable substrate, for **2** and **3** *versus* variable poly(dA)·$(T)_{10}$ at saturating TTP, and for **1** *versus* variable poly(dA)·$(T)_{10}$ at subsaturating TTP is that these photoprobes bind to both the free enzyme form of the Klenow fragment and the binary complex. These results suggest that the photoprobes bind to the Klenow fragment in the absence or presence of bound template-primer, similar to the activities of N^2-(4-*n*-butylphenyl)-dGTP at pol α (Khan et al., 1984). Additionally, at 20 μM, photoprobe **1** does not support polymerization catalyzed by the Klenow fragment using poly(dA-dT) as template-primer (data not shown), further evidence suggesting that this photoprobe forms an inhibition complex with the enzyme.

A more direct measure of the ability of the photoprobes to bind to the Klenow fragment in the absence of template-primer is in photoinactivation experiments. Photoinactivation of the Klenow fragment by **1** in the presence of a 200-fold excess of the nitrene scavenger β-mercaptoethanol suggests that this photoprobe does bind to the free enzyme form of the Klenow fragment and has a high proficiency as a nucleotide photolabel in this system. The control studies for photoinactivation of the Klenow fragment by photoprobe **1** demonstrate that inactivation requires 300 nm photolysis and that the Klenow fragment is stable in this time frame to photolysis in the absence of the photoprobe. However, in our hands, photolysis of the Klenow fragment for longer time periods at 300 nm led to significant inactivation, and the enzyme rapidly decomposed with photolysis at 276 nm.

Photoinactivation of the Klenow fragment by **1** was concentration-dependent and saturated at 9.7 μM, with an IC_{50} of about 2 μM, a result that correlates with the kinetic constant of 2.1 μM for inhibition of the Klenow fragment by **1** in competition with poly(dA)·$(T)_{10}$. This IC_{50} for photoinactivation of the Klenow fragment is 10−20 times the K_d for binding of dNTP to the Klenow fragment in the absence of template-primer (McClure & Jovin, 1975; Muise & Holler, 1985), evidence suggesting that the aryl azide and tether have a positive effect on enzyme binding activity. This increase in activity is consistent with our original suggestion that the tether length of minor groove-modified nucleotides may be an important determinant of enzyme binding activity. However, the absence of observable photoinactivation of the Klenow by **3** suggests that the aryl azide in this photoprobe does not make significant contact with the enzyme, so no conclusion can be made as to the efficacy with which this probe binds to the free enzyme. In contrast, photoprobe **2** readily cleaved under identical photoinactivation conditions, so failure to detect direct enzyme photoinactivation activity may be clouded by the cleavage reaction; we have not attempted nitrene scavengers other than β-mercaptoethanol.

It is of interest to note, however, that aryl azides photolyze to give a long-lived, electrophilic 1,2,4,6-cycloheptatetraene (also called dehydroazepine) intermediate which may be a poor label for ligands with weak binding affinity (Nielsen & Buchardt, 1982).

In conclusion, the adenosine-based photoprobes **1**−**3** have ideal properties for use as photoprobes of DNA polymerases, including rapid photodecomposition of the aryl azide at 300 and 350 nm with good quantum yield and stability of the adenosine nucleotide. Additionally, photoprobe **1** is a good inhibitor of the Klenow fragment, in both kinetic and photoinactivation studies. Although the three-atom tether length appears to be important for inhibition and photoinactivation activity, other polymerases may show differential selectivity, especially given that pol α prefers the one-atom tether between the guanosine and phenyl rings of N^2-(4-*n*-butylphenyl)-dGTP (Khan et al., 1984). In the following paper, we describe results demonstrating that photoprobe **1** is a specific and selective probe of the active site region of the Klenow fragment (Moore et al., 1996).

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