Van Stolk, B., & Noller, H. F. (1984) *J. Mol. Biol. 180*, 151–178.
Vasiliev, V. D., & Zalite, O. M. (1980) *FEBS Lett. 121*, 101–104.
Vasiliev, V. D., Selivanova, O. M., & Koteliansky, U. E. (1978) *FEBS Lett. 95*, 273–276.
Vogel, Z., Zamir, A., & Elson, D. (1968) *Proc. Natl. Acad. Sci. U.S.A. 61*, 701–707.
Zamir, A., Miskin, R., & Elson, D. (1971) *J. Mol. Biol. 60*, 347–364.
Zamir, A., Miskin, R., Vogel, Z., & Elson, D. (1974) *Methods Enzymol. 30*, 406–426.

Hydroxyl Radical Induced Cross-Linking between Phenylalanine and 2-Deoxyribose†

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ABSTRACT: Hydroxy radicals induce cross-linking between phenylalanine (Phe) and 2-deoxyribose (dR) via formation of corresponding free radical intermediates. The cross-linked products were separated and identified by capillary gas chromatography–mass spectrometry. When phenylalanine and 2-deoxyribose radicals were generated in a 1:1 ratio, the predominant interaction was between Phe and dR radicals while the Phe–Phe and dR–dR cross-links were less abundant. The newly discovered cross-link between 2-deoxyribose and phenylalanine may serve as a model for radiation or free radical induced cross-linking between DNA and proteins and in general between sugar moieties and amino acids.

The least studied and understood type of DNA damage in cells is the cross-linking between DNA and proteins (Oleinick et al., 1986). Radiation-induced cross-links were shown to occur in chromatin, in vivo and in vitro (Mee & Adelstein, 1981), and in mouse leukemia cells (Bowden et al., 1982). These cross-links have been suggested to result from free radicals of DNA and protein components generated in close proximity (Simic & Dizdaroglu, 1985). The most likely components of proteins to be involved in cross-linking would be aromatic and positively charged amino acids since they interact most intimately with DNA in the DNA–protein complex (Takeda, 1983).

Cross-linking mechanisms between thymine (T) and either phenylalanine (Phe) (Dizdaroglu & Simic, 1985) or tyrosine (Tyr) (Simic & Dizdaroglu, 1985) have been suggested on the basis of the observed T–Phe and T–Tyr cross-linked products. Participation of 2-deoxyribose (dR) in the DNA–protein cross-linking has not been considered so far mainly because of the inability to observe any radiation-induced sugar–sugar cross-links in model systems (Schulte-Frohlinde & von Sonntag, 1972; von Sonntag, 1987). In the present work, we find that hydroxy radical induced cross-links between phenylalanine and 2-deoxyribose can take place in model aqueous systems. It is suggested that this type of sugar–amino acid cross-link may be more relevant than base–amino acid cross-links in radiation-induced cross-linking between DNA and protein because of steric considerations associated with the DNA–protein interactions.

Materials and Methods

Materials.[1] Phenylalanine (ultrapure) was purchased from Vega Biochemicals and 2-deoxy-D-ribose (ultrapure) from Sigma. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine (ultrapure) were from Pierce. Water purified through a Millipore reverse osmosis system was used for all solutions.

Irradiations. Aqueous solutions of the mixture of Phe (0.5×10^{-3} M, pH 6.3) and dR (0.92×10^{-3} M) were saturated with oxygen-free N_2O (Matheson) for 30 min and irradiated in a ^{60}Co γ source (Woolf & Burke, 1984) (dose range 110–440 Gy, dose rate 110 Gy/min). Dose rate of the source was determined by using a Fricke dosimeter [$G(Fe^{3+}) = 15.6$; (Fricke & Hart, 1966)].

Trimethylsilylation. Samples (about 10 mg) dried with a rotary evaporator were trimethylsilylated (TMS) in Teflon-capped Hypovials (Pierce) with 0.1 mL each of BSTFA and pyridine (1:1) by heating for 30 min at 140 °C.

Gas Chromatography–Mass Spectrometry (GC–MS). A Hewlett-Packard Model 5880A microprocessor-controlled gas chromatograph interfaced to a Hewlett-Packard Model 5970A mass selective detector was used. The injection port and GC–MS interface were both maintained at 250 °C. Separations were carried out by using a fused-silica capillary column (12 m long × 0.2 mm i.d.) coated with cross-linked SE-54 (5% phenyl methyl silicon gum). Helium was used as the carrier gas at an inlet pressure of 100 kPa (split ratio 4:1, ion source temperature ca. 200 °C, electron energy 70 eV).

High-Performance Liquid Chromatography (HPLC). A Water Model 600 microprocessor-controlled liquid chromatograph equipped with a WISP Model 512 autoinjector and a Model 990 diode-array spectrophotometric detector was used. Separations were carried out on a 25 × 1 cm semiprep Supelcosil column SPLC-8-DB (particle size, 5 μm; Supelco) (eluent A, 0.1% trifluoroacetic acid (TFA) in water; eluent

† Taken from the Ph.D. dissertation of Mahnaz Farahani, Chemistry Department, The American University, Washington, DC 20016.

[1] The mention of commercial products is to provide technical information and is not intended as an endorsement.

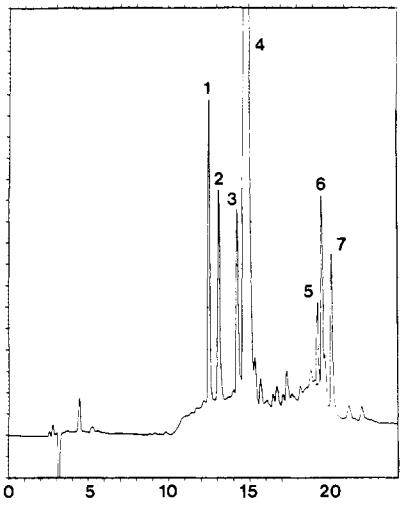


FIGURE 1: Liquid chromatogram obtained from γ-irradiated aqueous solutions of phenylalanine (Phe) and 2-deoxyribose (dR). Column: Supelcosil SPLC-8-DB (25 cm long × 1 cm; 5 μm). Eluent A, 0.1% TFA in water, eluent B, 0.1% TFA in acetonitrile. Elution program: isocratic for 10 min with eluent A, then linear gradient with a rate of 0.4% eluent B/min; flow-rate, 5 mL/min.

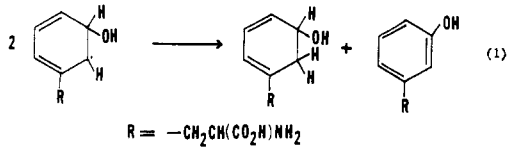
B, 0.1% TFA in acetonitrile).

Results and Discussion

Irradiation of aqueous solutions in the presence of N_2O generates OH radicals predominantly (92%) (Swallow, 1973). The OH radicals react rapidly with 2-deoxyribose and phenylalanie with rate constants of $k = 1.9 \times 10^9$ and 3.5×10^9 M^{-1} s^{-1}, respectively, as determined by pulse radiolysis (Farhataziz & Ross, 1977). The concentrations of Phe (0.5×10^{-3} M) and dR (0.92×10^{-3} M) were chosen according to their reaction rate constants in order to obtain an approximately 1:1 formation of Phe and dR free radical intermediates upon OH radical attack.

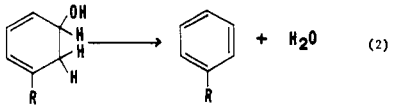
Reaction of OH radicals with compounds containing the benzene ring, such as Phe, is characterized by predominant addition to the ring (>90%) and the formation of the hydroxycyclohexadienyl radical (Dorfman et al., 1962). Abstraction of H from the α-position, though possible, should be a minor process for the zwitterion form of Phe due to decrease of the reactivity of the C–H bond by the protonated amino group (Simic, 1978).

Hydroxycyclohexadienyl radicals can disproportionate and dimerize. Disproportionation leads to formation of tyrosines.



R = $-CH_2CH(CO_2H)NH_2$

Hydroxycyclohexadienes are unstable and lose water, restituting Phe (Dizdaroglu & Simic, 1981).



Reaction of OH with 2-deoxyribose results in H abstraction at all five C positions and produces a series of products (Schulte-Frohlinde & von Sonntag, 1972). There were not indications of fragmentation of dR since products with a smaller number of carbon atoms were not detected. Hence, in the first step, H abstraction by OH gives C-1, C-2, C-3, C-4, and C-5 2-deoxyribose radicals. Approximately 50% of OH radicals abstract hydrogen from C-1 and C-2, about 20% from C-3 and C-4 each and only about 10% from C-5 (Schulte-Frohlinde & von Sonntag, 1972).

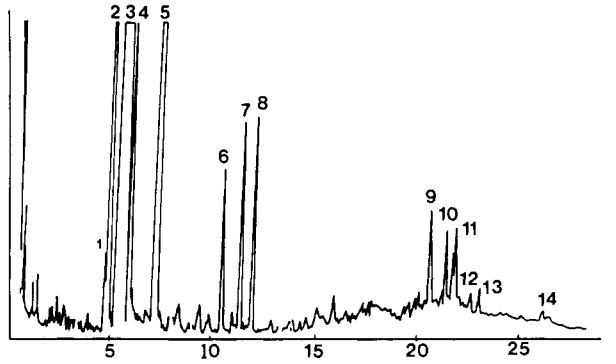


FIGURE 2: Gas chromatogram obtained from a γ-irradiated mixture of 2-deoxyribose and phenylalanine after trimethylsilylation. Column: fused-silica capillary column coated with cross-linked SE-54 (12 m long × 0.2-mm i.d.), programmed at 7 °C/min from 100 to 250 °C. Split ratio was 4:1. Column head pressure was 100 kPa.

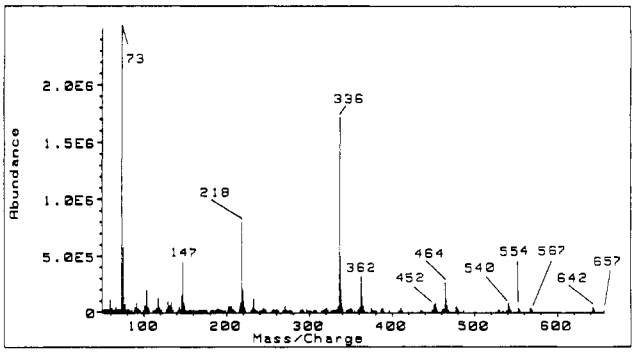
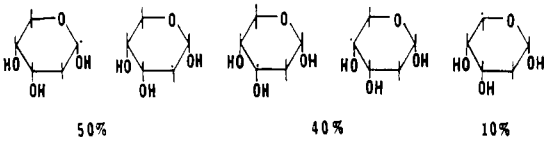


FIGURE 3: Mass spectrum of peak 9 in Figure 2.



Lyophilized samples were analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) with a solvent system consisting of 0.1% TFA in both water and acetonitrile (Mahoney et al., 1980). For this purpose ~10 mg of the sample was dissolved in 1 mL of water, 10–20 μL of which was injected into the liquid chromatograph. A typical chromatogram obtained from irradiated 2-deoxyribose + phenylalanine is shown in Figure 1.

Peaks 1, 2, and 3 in Figure 1 correspond to the radiation-induced products *p*-Tyr, *m*-Tyr, and *o*-Tyr, respectively. These peaks had the same retention times as those of the authentic materials. The formation of these products in the γ-radiolysis of Phe has been described before (Dizdaroglu & Simic, 1981). Peak 4 is L-Phe, and peaks 5–7 eluting after L-Phe correspond to Phe–dR dimeric products as characterized by GC–MS analysis of collected fractions.

Irradiated samples were also analyzed by capillary GC and GC–MS to characterize the dimers. Figure 2 shows a gas chromatogram obtained from irradiated 2-deoxyribose + phenylalanine after trimethylsilylation. Peaks 1–4 represent isomers of the TMS derivative of 2-deoxyribose. The retention time and mass spectrum for peak 5 are the same as that of the TMS derivative of authentic L-phenylalanine. Peaks 6, 7, and 8 in Figure 2 correspond to the radiation-induced

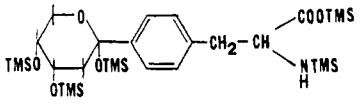
Table I: Product Yields (G-Values) in the Reaction of OH Radicals with Phenylalanine (0.5×10^{-3} M) and 2-Deoxyribose (0.92×10^{-3} M) in Aqueous Solutions and Molar Percent of OH in Each Product

peak in Figure 2	product	G-value	$G(OH)^a$	% OH[b]
6	*o*-tyrosine	0.39	0.78	13.93
7	*m*-tyrosine	0.38	0.76	13.57
8	*p*-tyrosine	0.32	0.64	11.43
9–12	dR–Phe	1.30	2.60	46.43
13–14	Phe–Phe	0.17	0.34	6.07

[a] Two OH radicals are needed for each molecular product, according to the proposed mechanism. [b] Percent of the total OH radicals produced [$G(OH)_t$ = 5.6] that are consumed to form the observed product.

monomeric products *o*-Tyr, *m*-Tyr, and *p*-Tyr, respectively, which have been reported previously (Dizdaroglu & Simic, 1985). Mass spectra taken from these peaks also were identical with those of authentic samples.

Similar mass spectra were obtained for peaks 9–12 in Figure 2. A representative mass spectrum is shown in Figure 3. Mass spectra were interpreted on the basis of known typical fragmentation patterns of similar compounds and their radiation-induced products as their trimethylsilylated derivatives. In all these spectra, molecular ion ($M^{\bullet+}$) and a characteristic $(M-CH_3)^{\bullet+}$ ion were observed at m/z 657 and 642, respectively. In the displayed spectrum, the ion at m/z 567 was due to elimination of (HOTMS:90) from $M^{\bullet+}$ ion. The ion at m/z 554 was due to elimination of (CH_2OTMS:103) from $M^{\bullet+}$ ion. Loss of COOTMS (amu 117) from $M^{\bullet+}$ accounted for the ion at m/z 540. The characteristic ion at 464 was due to elimination of (CH_2OTMS:103 and HOTMS:90) from $M^{\bullet+}$. The intense peak at 362 was due to elimination of (CH_2OTMS:103, CHOTMS:102, and HOTMS:90) from $M^{\bullet+}$. The intense peak at 336 was due to loss of 218 amu, a typical fragment for TMS derivatives of aromatic amino acids, and further loss of CH_2OTMS:103). The ion at m/z 218 is a result of loss of the aromatic side chain from the molecular ion. Ions of m/z 73 and 147 are commonly observed with TMS derivatives. On the basis of these data, this mass spectrum was attributed to the following representative compound (other isomers are also possible):



$M^+ = 657$

This compound, with a molecular ion m/z 657, is formed due to cross-linking of phenylalanine radical and 2-deoxyribose radicals. Peaks 13 and 14 were assigned to Phe–Phe dimers on the basis of mass spectra and retention times. The formation of these products in the OH reaction with L-Phe has been described (Simic et al., 1985).

Product Yields

Yields of the radiation-induced products were determined by GC using serine as an internal standard. The relative molar response factor (k value) of dR, Phe, and Tyr was found to be 1.02, 0.75, and 0.67, respectively, by using the method for the calculation of k values of trimethylsilylated compounds for FID (Ackman, 1964). The relative molar response factors for Phe–dR and Phe–Phe dimers were 0.89 and 0.75, respectively, which were calculated as the average of the relative molar response factors of the related monomers. The G-values (number of molecules formed/100 eV of radiation energy) of the products and OH radicals that are needed to generate them were calculated and are shown in Table I. Note that the G-values for *o*-, *p*-, and *m*-Tyr in this work are less than the G-values for these products in pure Phe (Simic et al., 1985) because of the competition of deoxyribose radical for the phenylalanine OH adducts.

Conclusion

From the data in Table I a material balance can be assessed. The total yield of OH radials is $G(OH)_t$ = 5.6, while only a fraction of those, $G(OH)$ = 5.1, leads to the formation of the observed products. Hence it appears that $G(OH) \sim 0.5$, which is not accounted for, will generate dR–dR dimers. These dimers were not observed under our experimental conditions, probably because of their inherent instability. The Phe–Phe and dR–Phe dimers, in contrast, are quite stable. On the basis of previously determined mechanisms for OH radical reactions with phenylalanine (Simic et al., 1985; Dizdaroglu & Simic, 1985) and 2-deoxyribose (Schulte-Frohlinde & von Sonntag, 1972; von Sonntag, 1987) and mechanisms for hetero-cross-links (Simic & Dizdaroglu, 1985; Dizdaroglu & Simic, 1985), the following mechanism is proposed for OH radical induced reactions in systems containing phenylalanine and 2-deoxyribose

$$\text{Phe} + {}^\bullet\text{OH} \rightarrow {}^\bullet\text{Phe–OH} \quad (3)$$

$$\text{dR} + {}^\bullet\text{OH} \rightarrow {}^\bullet\text{dR(–H)} + \text{H}_2\text{O} \quad (4)$$

$$2\,{}^\bullet\text{Phe–OH} \rightarrow \text{Phe(–H)–OH} + \text{PheH–OH} \quad (5)$$

$$\rightarrow \text{HO–Phe–Phe–OH} \quad (5a)$$

$$\text{HO–Phe–Phe–OH} \rightarrow \text{Phe(–H)–Phe(–H)} + 2\text{H}_2\text{O} \quad (6)$$

$$\text{PheH–OH} \rightarrow \text{Phe} + \text{H}_2\text{O} \quad (7)$$

$$2\,{}^\bullet\text{dR(–H)} \rightarrow \text{monomeric products} \quad (8)$$

$$\rightarrow \text{dR(–H)–dR(–H)} \quad (8a)$$

and finally the reaction that was the aim of our studies

$${}^\bullet\text{dR(–H)} + {}^\bullet\text{Phe–OH} \rightarrow \text{dR(–H)–Phe–OH} \quad (9)$$

followed by

$$\text{dR(–H)–Phe–OH} \rightarrow \text{dR(–H)–Phe(–H)} + \text{H}_2\text{O} \quad (10)$$

Hence, it is concluded that radiation (or free radical) induced cross-linking between DNA and proteins can take place not only between DNA bases (thymine) and aromatic amino acids but also between 2-deoxyribose and aromatic acids. The developed separation and measurement procedure may be utilized for isolation of dimeric products, such as dR(–H)–Phe(–H), and possibly for characterization of actual DNA–protein links.

References

Ackman, R. G. (1964) *J. Gas Chromatogr. 2*, 173.
Bowden, G. T., Kasunic, M., & Cress, A. E. (1982) *Radiat. Res. 89*, 203.
Dizdaroglu, M., & Simic, M. G. (1980) *Radiat. Res. 83*, 437.
Dizdaroglu, M., & Simic, M. G. (1981) *Oxygen Radicals*, pp 619–621, Academic, New York.
Dizdaroglu, M., & Simic, M. G. (1985) *Int. J. Radiat. Biol. 47*, 63–69.
Dorfman, L. M., Taub, L. A., & Buhler, R. E. (1962) *J. Chem. Phys. 36*, 3051.
Farhataziz & Ross, A. B. (1977) *Natl. Stand. Ref. Data Ser. (U.S., Natl. Bur. Stand.) NSRDS-NBS 59.*
Mahoney, W. C., & Hermodson, M. A. (1980) *J. Biol. Chem. 255*, 11199.
Mee, L. K., & Adelstein, S. J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 21194.

Nygaard, O. F., & Simic, M. G., Eds. (1983) *Radioprotectors and Anticarcinogens*, Academic, New York.
Oleinick, N. L., Chiu, S., Friedman, L. R., Xue, L. Y., & Ramakrishnan, N. (1986) in *Mechanisms of DNA Damage and Repair* (Simic, M. G., Grossman, L., & Upton, A. C., Eds.) pp 181-192, Plenum, New York.
Schulte-Frohlinde, D., & von Sonntag, C. (1972) *Isr. J. Chem. 10*, 1139.
Simic, M. G. (1978) *J. Agric. Food Chem. 26*, 6.
Simic, M. G., & Dizdaroglu, M. (1985) *Biochemistry 24*, 233.
Simic, M. G., Gajewski, E., & Dizdaroglu, M. (1985) *Radiat. Phys. Chem. 24*, 465-473.
Swallow, A. J. (1973) *Radiation Chemistry, an Introduction*, Wiley, New York.
Takeda, Y., Ohlendorf, D. H., Anderson, W. F., & Matthews, B. W. (1983) *Science (Washington, D.C.) 221*, 1020-1026.
von Sonntag, C. (1987) *The Chemical Basis of Radiation Biology*, Taylor & Francis, Philadelphia.
Woolf, S., & Burke, E. A. (1984) *IEEE Trans. Nucl. Sci. NS-31*, 1095-1097.

Functional Consequences of the Arabinosylcytosine Structural Lesion in DNA†

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ABSTRACT: Cytosine arabinoside (araC) is a potent antileukemic agent that is misincorporated into DNA in the course of its action. We have developed a chemical synthetic method that allows site-specific introduction of araC into synthetic DNA oligomers. We describe here the utilization of these oligomers as primer/template substrates for in vitro DNA synthesis reactions and as fragments for DNA ligation. These studies were undertaken to investigate the manner in which sites of araC misincorporation constitute sites of DNA dysfunction. AraCMP at the primer terminus dramatically reduced the rate of next nucleotide addition for *Escherichia coli* polymerase I (Klenow fragment) (Pol I), T4 polymerase, HeLa cell polymerase α_2 (Pol α_2), and AMV reverse transcriptase. Polymerases with associated 3′-5′ exonuclease activity preferentially excised araCMP from the primer terminus prior to chain elongation. AraCMP-terminated fragments were ligated more slowly than control fragments by T4 DNA ligase. AraCMP located at an internucleotide site in the template markedly slowed replicative bypass for Pol I, T4 polymerase, and Pol α_2, but not for reverse transcriptase. Synthesis was partially arrested after insertion of the correct nucleotide opposite the lesion site. These results suggest a complex mechanism for the inhibition of DNA replication by araC when it is misincorporated into DNA.

The nucleoside analogue 1-β-D-arabinofuranosylcytosine (araC;[1] Figure 1a) is an important agent in the treatment of various forms of leukemia (Frei et al., 1969; Bodey et al., 1969). Although araC is a formal analogue of the ribonucleoside cytidine (Figure 1b), differing only in configuration about the 2′ carbon, its metabolism more closely resembles that of deoxycytidine (Figure 1c). AraC is converted by a series of kinases to the active metabolite araCTP (Coleman et al., 1975; Hande & Chabner, 1978), which in vitro is an inhibitor of various DNA polymerases, notably DNA polymerase α (Momparler, 1972; Yoshida et al., 1977; Dicioccio & Srivastava, 1977), the putative replicative polymerase in mammalian cells. Kinetically, araCTP behaves as a competitive inhibitor with respect to dCTP (Furth & Cohen, 1968; Dicioccio & Srivastava, 1977). A possible mechanism for its inhibition of DNA synthesis involves misincorporation of the analogue at 3′ termini, followed by a slow or absent rate of addition of the next nucleotide (Momparler, 1972; Fridland, 1977; Cozzarelli, 1977).

At the cellular level, the major biochemical consequence of araC treatment is suppression of replicative (Graham & Whitmore, 1970; Heintz & Hamlin, 1983) and repair (Collins, 1977) DNA synthesis. Treatment of cells with araC results in misincorporation of the analogue into nuclear DNA primarily in internucleotide linkages, with increasing amounts found at chain termini at higher drug concentration (Major et al., 1981, 1982). The total amount of araC misincorporated into cellular DNA correlates very strongly with the cytotoxicity of the drug as measured by clonogenic assays (Kufe et al., 1980), suggesting, but not proving, a causal relationship between araC misincorporation and lethal cellular events.

The sites at which araCMP is misincorporated constitute loci of anomalous structure, or "lesions", in the DNA. Detailed understanding of how the presence of this lesion affects DNA replication processes should further elucidate the mechanism of araC toxicity. Moreover, study of the effects of DNA structural perturbations on the processes of replication may provide basic insights into the structural requirements for normal DNA replication.

Recently, we have developed methodology for chemical synthesis of DNA oligomers containing araCMP at specific sites (Beardsley et al., 1988). In this paper we describe the utilization of these oligomers as substrates for DNA polym-

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[1] Abbreviations: araC, 1-β-D-arabinofuranosylcytosine; DTT, dithiothreitol; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; PDE 2, phosphodiesterase 2; AMV, avian myeloblastosis virus; Pol α_2, HeLa cell polymerase α_2; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; TEAB, triethylammonium bicarbonate; TBE, buffer containing Tris, borate, EDTA, and urea.

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