Inactivation of *Escherichia coli* Ribonucleotide Reductase by 2′-Deoxy-2′-mercaptouridine 5′-Diphosphate. Electron Paramagnetic Resonance Evidence for a Transient Protein Perthiyl Radical[†]

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ABSTRACT: Ribonucleotide reductase catalyzes a key step in DNA biosynthesis and repair, supplying the cell with the four common deoxyribonucleotides. It is thus the target of antiproliferative agents. The enzyme consists of two subunits named protein R1 and protein R2. R1 provides the sites for the nucleotide substrates and redox-active cysteines required for catalysis. R2 harbors a tyrosyl radical essential for activity. We show here that 2′-deoxy-2′-mercaptouridine 5′-diphosphate, a substrate analog, is a very efficient inactivator of ribonucleotide reductase ($K_i = 35\ \mu M$, $k_{inact} = 0.18\ s^{-1}$). Inactivation is due to specific scavenging of the protein R2 tyrosyl radical. This unique feature sets this compound apart from other mechanism-based inhibitors such as 2′-azido- or 2′-chloro-2′-deoxyribonucleotide which induce partial or total protein R1 inactivation. During reaction, a transient organic radical was detected by EPR spectroscopy. Its *g* anisotropy ($g_z = 2.0620$, $g_y = 2.0265$, and $g_x = 2.0019$) and its hyperfine structure are consistent with a perthiyl RSS• radical. The loss of the hyperfine structure by deuterium labeling of the β protons of R1 cysteines unambiguously shows that the perthiyl radical is located on protein R1. We thus conclude that inactivation of ribonucleotide reductase by 2′-deoxy-2′-mercaptouridine 5′-diphosphate is due to an irreversible transfer of the radical located on protein R2 to a cysteine residue of protein R1.

DNA synthesis depends on a supply of deoxyribonucleotides. These are found at low levels within cells, and the enzymic reduction of ribonucleotides to deoxyribonucleotides is thought to be a rate-controlling step in the biosynthesis of DNA (Reichard, 1988; Eriksson & Sjöberg, 1989). The enzyme effecting this reduction, ribonucleotide reductase, is therefore a target in the design of antitumor agents and of antiviral agents (McCarthy & Sunkara, 1995; Robins et al., 1995).

The enzyme from *Escherichia coli* is the prototype for all mammalian and viral ribonucleotide reductases. It is composed of two homodimeric components designated R1[1] and R2 [see Stubbe (1990a) and Fontecave et al. (1992) for review]. R2 contains a dinuclear iron center and a stable tyrosyl radical essential for nucleotide reduction. Elegant studies from Stubbe strongly suggested that the tyrosyl radical generates, through long-range electron transfers, a thiyl radical in R1, thus making R1 active (Stubbe, 1990b; Mao et al., 1992a; Licht et al., 1996). R1 provides the binding site for the nucleoside diphosphate substrates (NDP) and binding sites for allosteric effectors. R1 contains five cysteines required for catalysis. C439 is proposed to be the protein thiyl radical on R1 present in the active site for initiating the reduction reaction by cleavage of the 3′-carbon−hydrogen bond of the nucleotide (Mao et al., 1992a) (Scheme 1). After protonation of the substrate radical and loss of a molecule of water, the intermediate radical cation is reduced by a pair of cysteines (C225 and C462) (Lin et al., 1987; Åberg et al., 1989; Mao et al., 1992b). This model is supported by the recent structure determination of the R1 subunit, which shows that the three cysteines C439, C225, and C462 are within 6 Å of one another (Uhlin & Eklund, 1994). The last substrate radical intermediate regains the hydrogen from C439, and the active site disulfide formed during the reduction of NDP to dNDP is reduced to a dithiol by a second pair of cysteines (C754 and C759) which receive electrons from thioredoxin.

The importance of ribonucleotide reductase in DNA synthesis has led to intensive studies on the inhibition of this enzyme. 2′-Substituted 2′-dNDP derivatives, as substrate analogs, have been shown to be very potent inactivators of *E. coli* ribonucleotide reductase, some of them with potential applications as anticancer drugs in clinics. Furthermore, studies with these inhibitors have provided much insight into the enzyme mechanism and confirmed the ability of the reductase to mediate radical-generating transformations (Stubbe & van der Donk, 1995).

In this work, we show that 2′-deoxy-2′-mercaptouridine 5′-diphosphate (**1**) (Scheme 2) is a very efficient inactivator of ribonucleotide reductase. The mechanism of the reaction has been studied and revealed unique features which set the compound apart from other substrate analogs, 2′-azido- and 2′-chloro-2′-deoxyribonucleotide. We report direct EPR detection of an enzyme cysteine-based radical upon mechanism-based inactivation of ribonucleotide reductase.

MATERIALS AND METHODS

CDP, ATP, dithiothreitol (DTT), and alkaline phosphatase from bovine intestine were from Sigma. Deuterium-labeled

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[1] Abbreviations: RDPR, ribonucleoside-diphosphate reductase; R1, protein component 1 of ribonucleotide reductase; R2, protein component 2 of ribonucleotide reductase; PFL, pyruvate formate-lyase; NDP and dNDP, ribonucleoside 5′-diphosphate and 2′-deoxyribonucleoside 5′-diphosphate.

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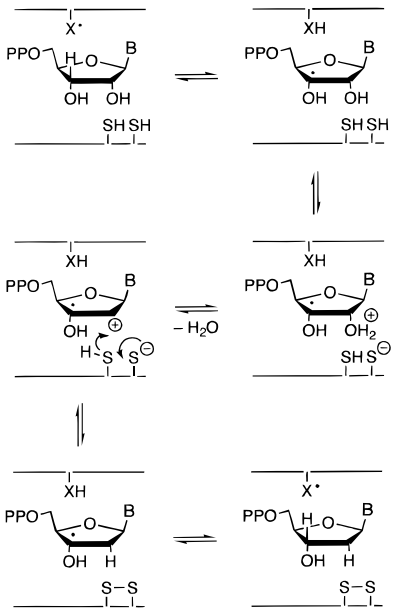
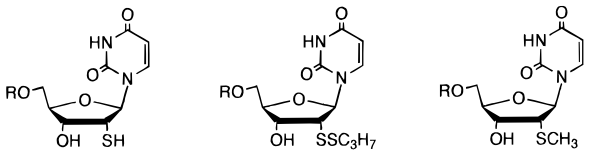
Scheme 1: Mechanism of Ribonucleotide Reduction by RDPR Adapted from Stubbe (1990a)[a]



[a] B represents base. A transient thiyl radical (S•) located on cysteine 439 of protein R1 abstracts a hydrogen atom from the 3′ position of the nucleoside diphosphate. H_2O is lost with subsequent oxidation of cysteines 225 and 462. The intermediate 2′-deoxy ribonucleotide radical is then reduced, regenerating the thiyl radical.

Scheme 2



R = PP: **1** R = H: **3** R = PP: **2** R = H: **4** R = PP: **5**

cystine (3,3,3′,3′-d_4, 98%) was purchased from Cambridge Isotope Laboratories. Tritiated CDP (specific activity, 629 GBq/mmol) was from Amersham. Deazaflavin (5-deaza-10-methylisoalloxazine) was synthesized according to a previously reported method (Yoneda et al., 1976).

All other chemicals were of the purest grade.

2′-Deoxy-2′-mercaptouridine 5′-diphosphate (**1**) was synthesized from 2′-deoxy-2′-mercaptouridine (**3**) prepared according to the literature (Imazawa et al., 1975; Divakar et al., 1990; synthesis and characterization of **1** will be described elsewhere). Briefly, after protection of the thiol group of the nucleoside as a mixed *n*-propyl disulfide, a tosyl group was introduced on the 5′-hydroxyl function of compound **4** and displaced by a pyrophosphate ion to obtain the diphosphate **2**. ^{1}H (500 MHz) and ^{31}P NMR (81 MHz) spectrometries showed that this compound was immediately reduced by DTT (1.1 equiv) to lead to the thiol diphosphate **1** exclusively. The structure of this compound was confirmed by TOCSY experiments in ^{1}H NMR spectrometry (500 MHz) and by high-resolution mass spectrometry.

E. coli AT2427 (*cysJ*43, *relA*1, *thi*-1, *spoT*1, λ^-, Hfr) is a *cysJ*$^-$ mutant strain obtained from Dr. B. J. Bachmann (*E. coli* Genetic Stock Center). Plasmids pTB1 and pGP1-2 were a kind gift from Professor Britt-Marie Sjöberg (University of Stockholm, Stockholm, Sweden).

Deuteriocysteine-labeled R1 protein (*d*-Cys-R1) was purified as previously described (Sjöberg et al., 1986) from *E. coli* strain AT2427, a cystein auxotroph, transformed with the plasmids pGP1-2 and pTB1. The latter is a recombinant derivative of pTZ18R containing the *nrdA* gene, encoding for the R1 protein.

A 50 mL preculture was grown overnight at 30 °C in minimal medium E supplemented with 0.2% glucose, 4 μg of thiamine, 200 μg/mL ampicillin, 40 μg/mL kanamycin, and 0.1 mM cystine-d_4 as the cysteine source. One liter of the same culture medium was then inoculated with 1% preculture and grown at 30 °C to an absorbance at 600 nm of ∼0.4. The temperature of the incubator was then shifted to 42 °C, and the cells were grown further to an absorbance of ∼1 prior to harvesting.

No growth could be observed in the absence of cystine, confirming the cysteine auxotrophy of *E. coli* AT2427 harboring *nrdA*.

E. coli ribonucleotide reductase proteins R1 and R2 were purified from overproducing strains as previously reported (Sjöberg et al., 1986; Larsson et al., 1988). Protein R1 mutants R1C439S, R1C225S, and R1C462S were a generous gift from Professor JoAnne Stubbe (Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA).

Protein concentration was estimated by the method of Bradford using bovine serum albumin as a standard (Bradford, 1976).

Inactivation of Ribonucleotide Reductase. RDPR activity was assayed in a final volume of 55 μL of 40 mM Tris/HCl (pH 8) containing 0.31 μM protein R1 and protein R2, 2 mM ATP, 10 mM DTT, 10 mM $MgCl_2$, 4 μg of thioredoxin, 2.3 mM [5-^{3}H]CDP, and increasing concentrations of substrate analog. After 10 min of incubation at 30 °C, reactions were stopped by placing the tubes in a boiling water bath for 1 min. The conversion of CDP to dCDP was measured as previously described (Steeper & Stuart, 1970). Control experiments were performed with no inhibitor in the incubation mixture.

Time-dependent inactivations were performed to investigate the inhibitory effect of 2′-deoxy-2′-mercaptouridine 5′-diphosphate (compound **1**). Protein R1 (0.5 μM) and protein R2 (0.5 μM) were preincubated at 30 °C in a final volume of 50 μL of Tris/HCl (pH 8) containing the mixture described above except that CDP was absent and the appropriate concentration of inhibitor was included. At time intervals, 2.5 μL of [5-^{3}H]CDP was added (final concentration, 2.3 mM) and the tubes were incubated again for 10 min at 30 °C for measuring the remaining RDPR activity. In control experiments, the inhibitor solution was replaced with water. The same experiment was also conducted in the absence of ATP during the preincubation time. In this case, ATP (final concentration, 2 mM) and CDP were added together in the second step of the assay.

Inhibition Specificity for Subunit R2. Protein R1 (2.5 μM) and protein R2 (2.5 μM) were incubated in a final volume of 50 μL of 40 mM Tris/HCl (pH 8) containing 10 mM DTT, 2 mM ATP, 17 μg of thioredoxin, 10 mM $MgCl_2$, and 25.7 μM compound **1**. An identical control was run with H_2O in place of the inhibitor.

After 30 min of incubation at 30 °C, a 5 μL aliquot was removed and added to 50 μL of the following mixtures for measuring the residual RDPR activity during 10 min at 30 °C: control, 40 mM Tris/HCl (pH 8), 10 mM $MgCl_2$, 2 mM ATP, 2.3 mM [5-^{3}H]CDP, 10 mM DTT, and 4 μg of thioredoxin; excess R1, same mixture as control plus 35 μg

of pure R1 protein; and excess R2, same mixture as control plus 12.7 μg of pure R2 protein.

Assays were run in triplicate in each case.

Activation of Ribonucleotide Reductase. RDPR (11 μM) was incubated in the presence of 110 μM compound **1** as described above for 30 min at 30 °C in a final volume of 0.4 mL. Controls were run with metR2 in place of R2 in the presence or in the absence of compound **1**. Then, each mixture was diluted to 1 mL with 50 mM Tris/HCl (pH 7.5) containing 20% glycerol and 10 mM $MgCl_2$ and extensively dialyzed at 4 °C in the same buffer. Proteins were concentrated by centrifugation on a Centricon-30 microconcentrator. After 10 min of photoactivation in the presence of 10 μM deazaflavin as previously described (Covès et al., 1995), a volume corresponding to 1 μg of R2 was assayed for RDPR activity in the presence of a saturating amount of protein R1.

Preparation of Oxidized Ribonucleotide Reductase. Oxidized RDPR was prepared as follows. R1 protein was first desalted by filtration on Sephadex G-25 equilibrated with 50 mM Tris/HCl (pH 7.5) containing 20% glycerol in order to remove DTT. This sample (3.1 mg of protein R1) was then mixed with 1.7 mg of protein R2 in a final volume of 500 μL of 50 mM Tris/HCl (pH 8) containing 10 mM $MgCl_2$, 1.5 mM ATP, and 0.6 mM CDP. After 10 min of incubation at 30 °C, the mixture was filtrated on a G-25 column equilibrated as described above. Protein fractions were pooled and concentrated on the Centricon-30 instrument.

Spectroscopic Methods. Scavenging of the tyrosyl radical was followed either spectrophotometrically, from the disappearance of the characteristic 410 nm absorption peak using a Kontron Uvikon 930 spectrophotometer, or by EPR spectroscopy. In this case, EPR first-derivative spectra were recorded at 100 K using a Bruker ESP 300E spectrometer operating at 9.41 GHz and equipped with a NMR gaussmeter. The microwave power was set at 2−10 mW, and the modulation amplitude was 0.31 mT.

Q-Band EPR spectroscopy measurements were performed at 100 K using a Varian Century-E-Line spectrometer operating at 35 GHz and equipped with a NMR gaussmeter. A 0.1% Varian Pitch in KCl was used as a reference.

EPR simulations were obtained with Frank Neese's program version 1.0 (University of Konstanz, Konstanz, Germany).

RESULTS

Inactivation of E. coli RDPR by 2′-Deoxy-2′-mercaptouridine 5′-Diphosphate. The *E. coli* ribonucleotide-diphosphate reductase (*E. coli* RDPR) activity of a stoichiometric R1/R2 mixture was assayed during a 10 min reaction in the presence of increasing concentrations of 2′-deoxy-2′-mercaptouridine 5′-diphosphate, compound **1**, and 2′-deoxyuridin-2′-yl *n*-propyldisulfide 5′-diphosphate, compound **2**. The structures of compounds **1** and **2** are shown in Scheme 2. Compound **2** served as a prodrug for **1**. It is highly stable in solution and prevents the sulfur group from autooxidation in air. Compound **1** is instantaneously generated from **2** during reaction with dithiothreitol (DTT), as shown by ^{1}H NMR spectrometry (data not shown). During this reaction, 1-propanethiol was formed and could be removed by bubbling argon through the solution. In the following experiments, compound **1** was prepared from **2**, just before addition to the reaction mixture.

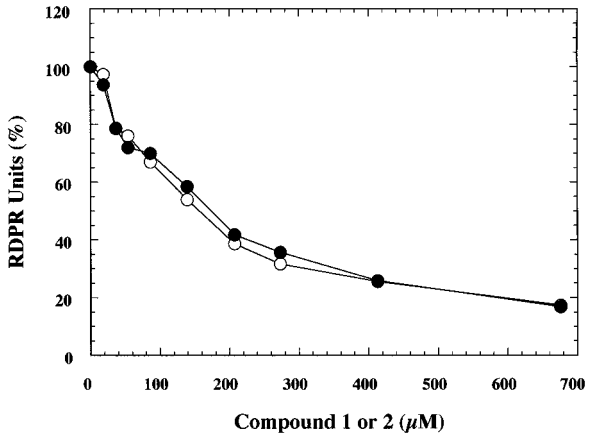


FIGURE 1: RDPR is inhibited by compounds **1** and **2**. RDPR activity was measured as described in Materials and Methods with increasing amounts of compound **1** (●) or compound **2** (○).

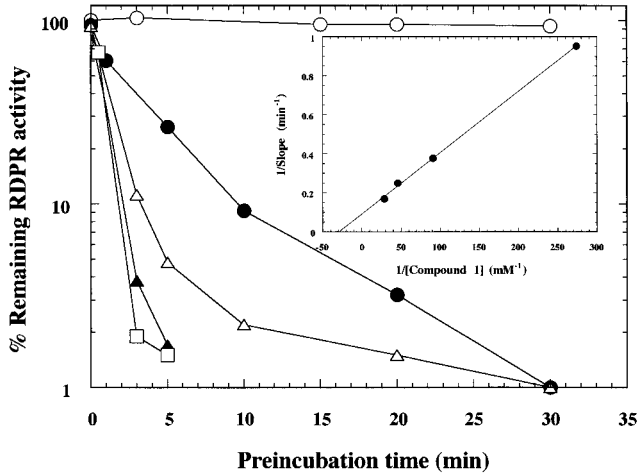


FIGURE 2: Time-dependent inactivation of RDPR by compound **1**. RDPR (0.5 μM) was incubated with various concentrations of compound **1**: (○) 0 mM, (●) 3.65 μM, (△) 11 μM, (▲) 21.8 μM, and (□) 35 μM. At the indicated times, CDP was added and the remaining activity measured. The inset is a replot of the reciprocals of the slopes vs the reciprocals of the inhibitor concentrations: K_i = 35 μM and k_{inact} = 0.18 s^{-1}.

As shown in Figure 1, compounds **1** and **2** had comparable inhibitory effects on the enzymatic reduction of 1.5 mM CDP with an IC_{50} of approximately 150 μM. The activity of compound **2** is due to its rapid conversion into **1** during the assay, since the reaction mixture contained 10 mM DTT, as an electron source.

2′-Deoxy-2′-mercaptouridine, compound **3**, the nucleoside analog of **1** lacking the diphosphate group, and compound **4**, the corresponding nucleoside analog of **2** were totally inactive. **3** was prepared from **4** by reduction with DTT. The strict requirement for the diphosphate group indicated that compound **1** binds to the substrate site as a prerequisite for inactivation. We also tested compound **5**, 2′-deoxy-2′-methylthiouridine 5′-diphosphate (Scheme 2), and found no inhibitory effects on CDP reduction by RDPR.

Incubation of varying concentrations of compound **1** with *E. coli* RDPR in the presence of ATP but in the absence of substrates and then assaying for enzymatic activity by dilution into a solution containing a large excess of CDP resulted in the time-dependent inactivation shown in Figure 2. Again, under those conditions, compounds **3** and **4** could not inactivate RDPR (data not shown). A replot of the data (inset of Figure 2) gave K_i = 35 μM and k_{inact} = 0.18 s^{-1}.

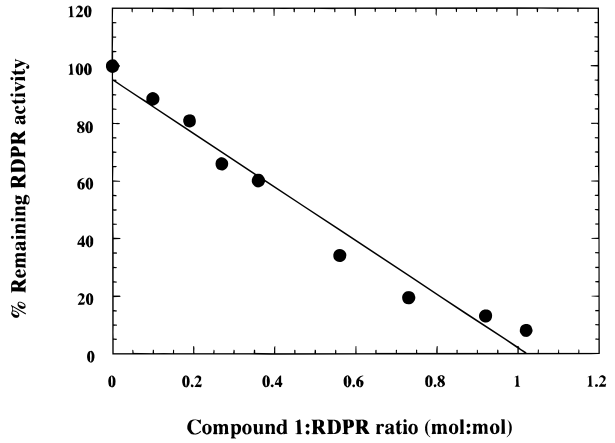


FIGURE 3: Inactivation of RDPR by compound **1** is a stoichiometric reaction. RDPR (0.5 μM) was incubated during 110 min with various concentrations of compound **1** in the absence of the substrate. Then CDP was added and the remaining activity measured.

The concentration dependence on the inactivation indicates a very high affinity of the enzyme for **1**, in the same range as that for the corresponding natural substrate, uridine 5′-diphosphate, UDP ($K_m \sim 200$ μM). Moreover, comparison of Figures 1 and 2 shows that CDP protects the enzyme from inactivation by compound **1**, confirming that binding of the analog is required for inactivation and that the same site binds both CDP and **1**. As a matter of fact, RDPR was totally inactivated during 5 min of incubation with 22 μM **1** (Figure 2), while in the presence of 1.5 mM CDP, still 50% activity was retained after 10 min of incubation with 150 μM **1**. This model is further supported by the observation (not shown) that ATP was absolutely required, in the preincubation mixture, for the inactivation of RDPR by compound **1** to occur. ATP, a positive effector for binding and reduction of UDP and CDP, thus also increased the affinity of the reductase for compound **1**.

The efficiency of the inactivation of RDPR by compound **1** was determined by partially inactivating the enzyme with various amounts of the inhibitor. The remaining activity was measured after 110 min of incubation. Figure 3 demonstrates that the inactivation was extremely efficient, with only 1 equiv of **1** capable of eliminating RDPR activity almost completely. The lack of multiple turnovers thus makes very difficult the isolation, identification, and quantitation of the small amount of products formed in the reaction. Such a study will require the preparation of isotopically labeled compounds.

2′-Deoxy-2′-mercaptouridine 5′-Diphosphate Selectively Scavenges the Tyrosyl Radical of R2 and Converts R2 into metR2. The inactivation of RDPR is irreversible, and no recovery of enzymatic activity was observed after passing the inactivated enzyme mixture through a Sephadex G50 column. To determine which of protein R1 and protein R2 was the site of the inactivation, the following experiment was carried out.

After inactivation of 2.5 μM RDPR with 25.7 μM compound **1**, an aliquot of the reaction mixture was removed and diluted in a CDP reductase assay mixture containing either no additional protein or a saturating excess of protein R1 or protein R2. Activities were compared to the CDP reductase activity from a control experiment in which only compound **1** was absent. The results are shown in Table 1. It is clear that only in the case of complementation of the assay with protein R2 was the activity restored. Thus, compound **1** selectively inactivates R2. R1 might also have been modified but with no effect on the activity.

Table 1: Selective Inhibition of Protein R2 by Compound **1**[a]

	RDPR activity (nmol of CDP reduced/min)
control (0.25 μM RDPR)	3
+ excess R1	8[b]
+ excess R2	3.4
control + compound **1**	0.04
+ excess R1	0.07
+ excess R2	3.7

[a] 2.5 μM RDPR was incubated during 30 min at 30 °C in the presence of 25.7 μM compound **1** as described in Materials and Methods. Then, a 5 μL aliquot was added to 50 μL of RDPR assay mixture containing either an excess of protein R1 (35 μg) or protein R2 (12.7 μg). Controls were run under the same conditions in the absence of added protein R1 or R2. [b] The increase of enzyme activity reflects that it is not measured under saturating conditions when stoichiometric amounts of R1 and R2 are assayed.

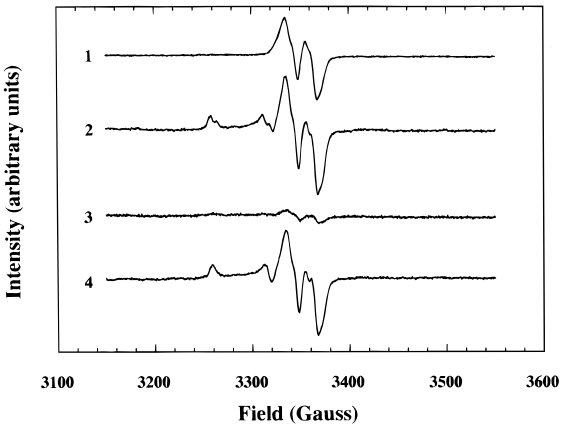


FIGURE 4: X-Band EPR spectra obtained after addition of compound **1** to RDPR. Spectrum 1 shows the starting RDPR tyrosyl radical. Spectra 2 and 3 were recorded after 45 s and 3 min of incubation, respectively, of 11.5 μM RDPR with a 10-fold excess of compound **1**. Spectrum 4 was obtained also after 45 s of incubation, but RDPR consisted of R2 and deuteriocysteine-labeled R1. General conditions are as follows: temperature, 100 K; modulation amplitude, 0.31 mT; and time constant, 10 ms. Specific conditions are as follows: (spectrum 1) microwave power, 10 mW; and microwave frequency, 9.409 19 GHz; and (spectra 2−4) microwave power, 2 mW; and microwave frequencies, 9.412 26, 9.4102, and 9.410 25 GHz, respectively. They are a 3-fold magnification of real spectra.

To determine whether the essential tyrosyl radical of protein R2 was a site for the inactivation, the reaction of 11.5 μM RDPR with 100 μM compound **1** in the presence of ATP was monitored by EPR spectroscopy. The EPR signal characteristic of the tyrosyl radical of protein R2 (Figure 4, spectrum 1) disappeared within a few minutes. After 3 min, the reaction mixture was EPR silent (Figure 4, spectrum 3). Scavenging of the tyrosyl radical was also observed by UV−visible spectroscopy, from the disappearance of the 410 nm absorption peak, characteristic of the radical. This experiment also showed that the iron center was not altered since the 370 nm absorption band, characteristic of the Fe−O−Fe unit, was unchanged during the reaction (data not shown). The inactive R2 was thus suspected to be the well-known metR2 form, which has a normal tyrosine residue and an intact iron center. One of the characteristics of metR2 is that it can be reactivated during incubation with photoreduced deazaflavin plus O_2 (Covès et al., 1995). As shown in Table 2, this also

Table 2: Inactivation of RDPR Is Reversible[a]

protein conditions	RDPR activity (nmol of CDP reduced/min)
R1 − R2 + compound **1**	6.7
R1 − metR2 + compound **1**	6.5
R1 − metR2	6.7

[a] 11 μM RDPR was incubated during 30 min at 30 °C in the presence of 110 μM compound **1** as described in Materials and Methods. As a control, protein R2 was replaced with the same amount of metR2. Another control containing metR2 was run under the same conditions except that compound **1** was omitted. After dialysis and concentration as specified in Materials and Methods, RDPR was photochemically activated in the presence of 10 μM deazaflavin. In each case, a volume corresponding to 1 μg of protein R2 was assayed for activity in the presence of a saturating amount of protein R1. Assays were run in triplicate.

happened to be the case with the R2 form inactivated by compound **1**.

A Radical Intermediate in the Reaction between RDPR and 2′-Deoxy-2′-mercaptouridine 5′-Diphosphate. By monitoring the inactivation reaction by EPR spectroscopy at 100 K, we could observe the transient formation of an unstable radical species (Figure 4, spectrum 2). A new EPR signal appeared soon after addition of compound **1** to the reaction mixture and was detectable all during the course of the reaction, while the EPR signal of the tyrosyl radical rapidly decayed. No other signals could be detected in the field scan range 100−400 mT. Both signals disappeared together at the end of the reaction after 3 min of incubation (Figure 4, spectrum 3). When protein R1 was omitted, the tyrosyl radical of protein R2 was stable and was the only radical to be detectable by EPR spectroscopy. Furthermore, protein R2 remained active, showing that tyrosyl radical scavenging reaction and inactivation of protein R2 by compound **1** and formation of the new radical species absolutely required protein R1. Figure 5A (spectrum 1) shows the EPR spectrum of the transient radical after subtraction of the remaining tyrosyl radical spectrum from spectrum 2 of Figure 4. This operation was possible since the tyrosyl radical EPR spectrum was not significantly changed, apart from its intensity, during the reaction. Moreover, it was crucial to fit the microwave frequency of the tyrosyl radical spectrum (spectrum 1 of Figure 4) to the frequency of spectrum 2 of Figure 4. The isotropy of the **g** tensor of the tyrosyl radical ($g_x = 2.002\,00$, $g_y = 2.004\,23$, and $g_z = 2.008\,66$) makes this possible, and errors on field values will be less than 10^{-4} mT if the calculations used $g_{av} = 2.004\,96$ and a field translation. After frequency correction, subtraction was done for three different samples and the same signal was always obtained (Figure 5A, spectrum 1). This signal exhibits three clearly resolved doublets (splittings of *ca*. 18.3 MHz) centered at the calculated *g* values $g_z = 2.0620$, $g_y = 2.0265$, and $g_x = 2.0019$. The small feature at 3345 G is an artifact of the subtraction operation. The properties of the EPR signal (temperature dependence, power saturation) were characteristic of a free organic radical.

In order to analyze this species more thoroughly, an EPR study at Q-band frequencies was performed (data not shown). This experiment clearly proved that the shape of the spectrum was dominated by *g* anisotropy and that the three doublets actually corresponded to three different *g* values with a hyperfine splitting nearly isotropic. All other analyses with large hyperfine couplings could therefore be rejected. It became thus possible to simulate spectrum 1 of Figure 5A

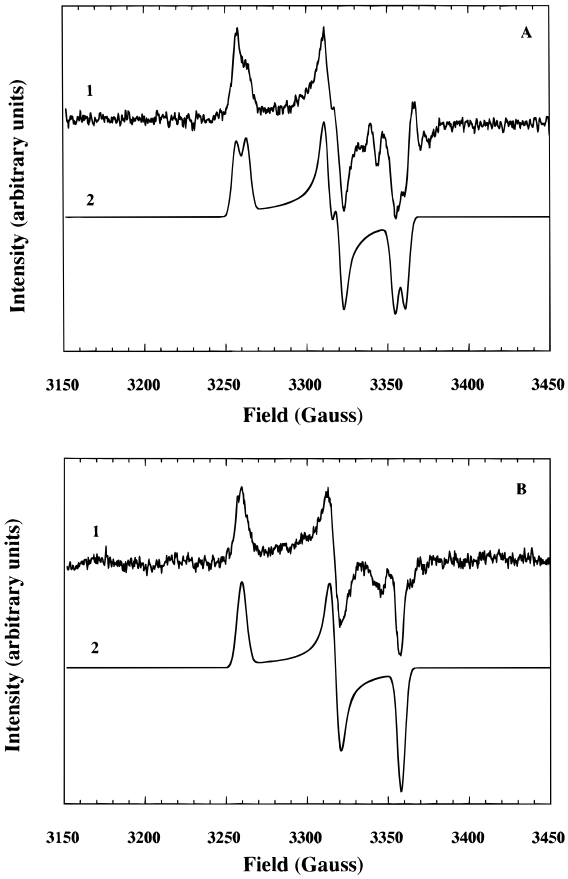


FIGURE 5: EPR spectrum of the transient radical. (A) Spectrum 1 (native RDPR) was obtained by subtracting the signal of the remaining tyrosyl radical from spectrum 2 of Figure 4 after frequency correction. Spectrum 2 is a simulation of spectrum 1 using the EPR parameters listed in Table 3. (B) Spectrum 1 (RDPR containing deuteriocysteine-labeled R1) was obtained by subtracting the signal of the remaining tyrosyl radical from spectrum 4 of Figure 4 after frequency correction. Spectrum 2 is a simulation of spectrum 1 using the EPR parameters cited above. EPR conditions are as follows: temperature, 100 K; modulation amplitude, 0.31 mT; microwave power, 2 mW; time constant, 10 ms; and microwave frequencies, 9.412 27 and 9.410 25 GHz for panel A and B, respectively.

using the *g* values and hyperfine splitting constants listed in Table 3. The simulated spectrum is displayed in Figure 5A (spectrum 2).

Oxygen Is Required for the Observation of the Radical Intermediate. In studies aimed at understanding the nature of the transient radical species, we looked at the role of oxygen in the inactivation reaction. We thus made the unexpected observation that, under anaerobic conditions, the EPR spectrum of the reaction mixture containing RDPR and compound **1** was still unchanged after 15 min of reaction (while it has completely disappeared after 3 min under aerobic conditions). In the absence of oxygen, the tyrosyl radical is thus not destroyed and the previously observed transient radical species not formed. However, when later oxygen was introduced into the reaction mixture, the intensity of the tyrosyl radical signal started to decline and the new EPR signal appeared. If anaerobiosis is maintained further, decrease or even loss of inhibition is observed upon reoxygenation.

Effects of Mutations at Redox-Active Cysteines of R1 on the Radical Intermediate. Recent site-directed mutagenesis studies have suggested that C225 and C462 are the cysteines directly involved in nucleotide reduction (Mao et al., 1992b).

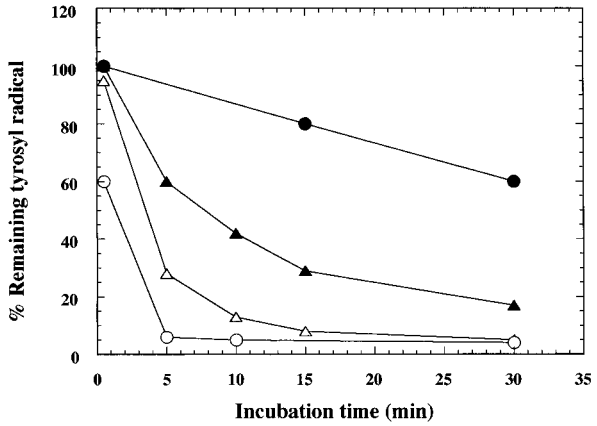


FIGURE 6: Effects of mutations at the redox-active cysteines of protein R1 on R2 inactivation by compound **1**. Mixtures containing different preparations of protein R1 were incubated with 1 equiv of protein R2 and a 10-fold excess of compound **1** at 30 °C as described in Materials and Methods. At time intervals, R2 tyrosyl radical content was quantified by EPR spectroscopy. The RDPR concentration was 11.5 μM in each case. Reaction mixtures contained R1C462S (△), R1C225S (▲), fully oxidized native R1 (●), and reduced native R1 (○). One hundred percent tyrosyl radical corresponds to a freshly prepared R2 protein containing 1.2 tyrosyl radicals per dimer.

To address whether either of the active site cysteines was involved in the reaction producing the new radical, compound **1** was added to mixtures containing R2 and mutant R1 proteins, C225SR1 and C462SR1, under the same conditions as for native R1. As with native R1, the loss of the tyrosyl radical of protein R2 was promoted by mutant R1 proteins, albeit at a slower rate (Figure 6). However, no evidence for the anisotropic signal previously observed could be obtained. Therefore, while active site cysteines 225 and 462 are not required for scavenging the tyrosyl radical by compound **1**, they are required for the production/stabilization of the second radical species. This is in agreement with the observation that inactive oxidized RDPR, i.e. containing cysteines 225 and 462 as a disulfide, supported the loss of the tyrosyl radical, although at a reduced rate and with no formation of an intermediate radical species, in the absence of DTT/thioredoxin (Mao et al., 1992b). Finally, cysteine 439 was found to be absolutely required for the scavenging of the tyrosyl radical by compound **1**. As a matter of fact, the C439SR1 mutant protein, in which cysteine 439 was changed into a serine, supported a very slow loss of the tyrosyl radical, which accounted for the 3% wild type R1 contaminant (data not shown), due to the expression system (Mao et al., 1992a).

This led us to study the effect of specific deuterium labeling of the β-methylene hydrogens of cysteines of R1. Labeled R1 was prepared by protein expression in *E. coli* AT2427, a cystein-requiring auxotroph, grown in the presence of [β-^{2}H]cysteine. The resulting X-band EPR spectrum of a reaction mixture containing compound **1**, R2, and labeled R1 is shown in Figure 4 (spectrum 4), and the spectrum of the radical intermediate after subtraction of the remaining tyrosyl radical is shown in Figure 5B (spectrum 1). It is readily apparent that the proton hyperfine interaction has been eliminated, especially if one compares the low-field feature of the signals generated with native R1 (doublet signal) and labeled R1 (singlet signal). Spectrum 1 of Figure 5B could be correctly simulated with $g_z = 2.00624$, $g_y = 2.0625$, and $g_x = 2.0019$ (Figure 5B, spectrum 2), in perfect agreement with the parameters determined for unlabeled R1. This establishes unambiguously that the intermediate radical is a protein radical with the spin density located on a cysteine residue of protein R1 and that the unpaired electron spin is coupled to the β-hydrogen of the residue.

DISCUSSION

The chemical group X at the 2′ position of the ribose moiety of a ribonucleoside diphosphate provides a crucial control for its reduction by RDPR (Stubbe & van der Donk, 1995). With natural substrates (X = OH), the reduction by C225 and C462 proceeds and follows the mechanism shown in Scheme 1 to generate 2′-deoxyribonucleotides. After one catalytic cycle, protein R2 regains its tyrosyl radical and no inactivation of RDPR is observed. When X = Cl or X = F, a rapid time-dependent inactivation of protein R1 is initiated by 3′-carbon−hydrogen bond cleavage (Stubbe, 1990a). However, as for natural substrates, the tyrosyl radical is reduced and regenerated during one inactivation cycle and no evidence was obtained for inactivation of protein R2 and irreversible scavenging of its tyrosyl radical. When X = N_3, a different mechanism takes place, with partial inactivation and covalent modification of protein R1 and with complete destruction of the tyrosyl radical of protein R2, again initiated by 3′-carbon−hydrogen bond cleavage (Salowe et al., 1993). The most interesting result is that the reaction results in production of a stable radical intermediate concomitant with loss of the tyrosyl radical, thus providing the first direct confirmation that RDPR mediates radical-generating transformations (Sjöberg et al., 1983). Extensive studies using a variety of isotopically labeled derivatives have suggested that the radical is nitrogen-centered and covalently bound to cysteine 225 (van der Donk et al., 1995).

The results presented here demonstrate that when X = SH, *E. coli* RDPR is inactivated with a third type of mechanism. As a matter of fact, only protein R2 activity was destroyed; only addition of pure active R2 to RDPR inactivated by compound **1** could restore ribonucleotide reductase activity, while addition of R1 was without effect. These features set compound **1** apart from other 2′-substituted 2′-deoxyribonucleotides as mechanism-based inhibitors, since it is the first example in which protein R1 is not partially or totally inactivated.

It is quite clear that the inactivation reaction resides on the stoichiometric one-electron reduction of the tyrosyl radical of protein R2 and the transformation of R2 into metR2, an inactive form lacking the radical but still containing the iron center. EPR and UV−visible spectroscopies provide the direct evidence for the loss of the radical and the persistence of the iron center. Moreover, the R2 form inactivated by compound **1** can be reactivated during reduction and reoxidation by O2, in the absence of exogenous iron, conditions used to reactivate metR2 (Covès et al., 1995).

It is very interesting to thus define compound **1** just as a tyrosyl radical scavenger, with a global reactivity comparable to that of hydroxyurea, an anticancer agent used in clinics. The antiproliferative effects of hydroxyurea are mainly due to its ability to scavenge the tyrosyl radical of ribonucleotide reductase (Lassman et al., 1992). However, compound **1** is much more active than hydroxyurea because, as a substrate analog, it is designed to interact both strongly and selectively with the enzyme, which makes it a perfect specific tyrosyl radical scavenger. The efficacy of the inhibitory properties of compound **1** can be appreciated from (i) its ability to inactivate RDPR in a stoichiometric reaction; (ii) the low K_i

Table 3: Comparison of EPR Parameters of Previously Reported Perthiyl Radicals

compound	tem (K)	g_{av}	g_z	g_y	g_x	a (mT)	references
RSS$^\bullet$ (RDPR)	100	2.030	2.062	2.0265	2.0019	0.645 (1H)	this work
RSS$^\bullet$ (PFL)	77	2.027	2.057	2.023		1.0 (1H)	Parast et al., 1995
MeSS$^\bullet$	77	2.027	2.056	2.025	2.000	0.72 (3H)	Nelson et al., 1977
EtSS$^\bullet$	77	2.029	2.059	2.026	2.002	0.8 (1H)	Nelson et al., 1977
$^+H_3NCH_2CH_2SS^\bullet$,Cl^-	77	2.028	2.058	2.025	2.001	(1H)	Nelson et al., 1977
crystal	77	2.029	2.054	2.027	2.005	0.9 (1H)	Henriksen, 1962
$HO_2CCH_2CH_2SS^\bullet$	77	2.029	2.061	2.025	2.001	0.9 (1H)	Nelson et al., 1977
$HO_2CCH(CH_3)SS^\bullet$	77	2.028	2.059	2.025	2.000		Nelson et al., 1977
$HO_2CCH(NH_3^+)CH_2SS^\bullet$,$Cl^-$							
crystal (I)	77	2.026	2.053	2.026	2.000	1.0 (1H)	Hadley & Gordy, 1974
crystal (II)	77	2.032	2.067	2.027	2.002	0.5 (1H)	Hadley & Gordy, 1974
	77	2.026	2.055	2.024	2.000	1.0 (1H)	Nelson et al., 1977
crystal (I)	77	2.025	2.055	2.023	1.998	1.1 (1H)	Hadley & Gordy, 1975
crystal (II)	77	2.028	2.062	2.023	1.999	0.5 (1H)	Hadley & Gordy, 1975
crystal (free base)	77	2.028	2.052	2.025	2.006	0.9 (1H)	Henriksen, 1962
$HO_2CCH(NH_3^+)C(CH_3)_2SS^\bullet$,$Cl^-$	77	2.028	2.057	2.025	2.001		Nelson et al., 1977
crystal (free base)	77	2.028	2.053	2.026	2.004		Henriksen, 1962
$ClCH_2CH(OH)CH_2SS^\bullet$	77	2.028	2.059	2.025	2.001	0.8 (1H)	Nelson et al., 1977
$HSCH_2(CHOH)_2CH_2SS^\bullet$	77	2.029	2.060	2.025	2.001	0.9 (1H)	Nelson et al., 1977

value ($K_i = 35\ \mu M$) of the enzyme for compound **1**, which is one of the lowest values reported so far for a uridine analog; and (iii) the high k_{inact} value ($k_{inact} = 0.18\ s^{-1}$), reflecting a much faster inactivation with compound **1** than with 2′-deoxy-2′-methyleneuridine (Baker et al., 1991) or with 2′-azido-2′-deoxyuridine (Salowe et al., 1993) for example.

The following results clearly show that the inhibitor must bind to the active site of the reductase to be effective. (i) The substrate CDP protects from inhibition. (ii) Inhibition absolutely depends on the presence of ATP, a specific positive effector for the reduction of UDP and CDP. (iii) The nucleoside analog, compound **3**, lacking the diphosphate group, has no inhibitory effects and no tyrosyl radical scavenging properties. (iv) R1 is absolutely required for the reaction between R2 and compound **1** to occur. 2′-Mercapto-2′-deoxyuridine 5′-diphosphate is thus a specific hydroxyurea-like scavenger of the tyrosyl radical of R2, only in the presence of protein R1. Protein R1 serves to bind the inhibitor, to present it to protein R2 correctly, and to mediate the electron transfer from the nucleotide to the radical. We assume that cysteine 439 is mediating the reaction, as for natural substrates. As a matter of fact, mutation of cysteine 439 to serine totally blocks this electron transfer.

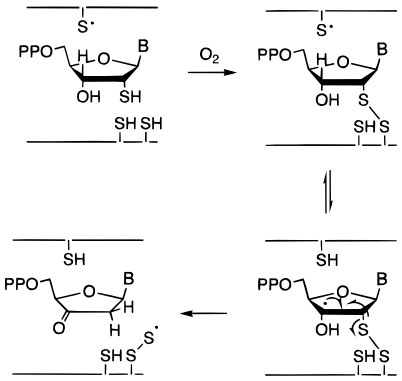
The most intriguing result is the observation that the loss of the tyrosyl radical was accompanied by transient formation of a new unstable organic radical species, characterized by a three-line EPR spectrum and 6.45 G doublet splittings. As unambiguously demonstrated by the recording of an EPR spectrum at Q-band frequencies (35 GHz), the shape of the signal is not due to hyperfine couplings (to an $I = 1$ nucleus) but instead reflects g anisotropy. We were able to simulate the spectrum correctly with the following g values: $g_z = 2.0620$, $g_y = 2.0265$, and $g_x = 2.0019$ ($g_{av} = 2.0302$).

These g values and hyperfine splittings are closely related to those reported for perthiyl radicals (RSS$^\bullet$) from cysteamine and cysteine derivatives (Table 3 and references therein). During this work, a comparable EPR spectrum has been reported and was assigned to a perthiyl radical presumably located on the cysteine 418 residue of pyruvate formate-lyase (Parast et al., 1995). This radical was formed during inactivation of PFL by mercaptopyruvate. Sulfinyl radicals (RSO$^\bullet$), disulfide radical cations (RSSR$^{\bullet+}$), disulfide radical anions (RSSR$^{\bullet-}$) (Bassindale & Iley, 1990), and peroxyl radicals (ROO$^\bullet$) (Sevilla et al., 1990) can be ruled out on the basis of their **g** tensor and their too small g_{av} values (2.010−2.020).

As a consequence, we suggest that the transient radical formed during inactivation of RDPR by compound **1** is a perthiyl radical, located on a cysteine residue of protein R1. This is shown unambiguously from the effect of deuterium labeling of R1 cysteines on the EPR spectrum (loss of the 6.45 G hyperfine coupling doublet structure). To account for the apparent doublet hyperfine interaction, the geometry of one of the β-hydrogens of the cysteine must be ~90° with respect to the orbital of the unpaired electron on the sulfur atom such that its coupling constant is very small. A previous study of perthiyl radicals formed during irradiation of cysteine or cystine has indeed shown that splitting is resolved for only one β-hydrogen nucleus (Hadley & Gordy, 1975). An unresolved question is which cysteine carries the radical. Both single cysteine to serine mutations at positions 225 and 462 prevent the formation of the perthiyl radical, and thus, both cysteines are candidates. A likely possibility is that only one of them is the radical site but mutation of the other affects the proper orientation of the reactants and the reaction between the nucleotide and the active cysteine. Another likely possibility is that the perthiyl radical has become undetectable by EPR spectroscopy as a consequence of a decreased steady state concentration due to the mutations. It should be mentioned that cysteine 225 is the transient nitrogen-centered radical site during inactivation of RDPR by 2′-azido-2′-deoxyuridine (van der Donk et al., 1995).

The mechanism of formation of the sulfur-based radical is speculative at this point, and several alternatives are probably possible. A likely possibility is that 2′-deoxy-2′-mercaptouridine 5′-diphosphate acts as a mechanism-based inhibitor (Scheme 3). Upon binding of compound **1** to the active site of RDPR, a mixed disulfide is generated, coupling the sulfur atom of the substrate analog to a cysteine of R1, in the presence of oxygen. As for the natural substrate, C439 thiyl radical, generated at the expense of the R2 tyrosyl radical, then abstracts the 3′-hydrogen of the mixed disulfide intermediate. The next step is proposed to be the homolytic cleavage of the 2′-carbon−sulfur bond, releasing a 3′-keto derivative and generating the perthiyl radical observed by EPR spectroscopy. Such a β cleavage, generating thiyl radicals, is well-documented (Boothe et al., 1978). The tyrosyl radical cannot be regenerated during reaction of the perthiyl radical with protein R2, thus explaining the inactiva-

Scheme 3: Proposed Mechanism-Based Inactivation of RDPR by Compound **1**[a]



[a] B represents uracil. In the presence of oxygen, a mixed disulfide is generated between the substrate analog and the protein. Thiyl radical then abstracts the 3′-hydrogen atom. Generation of the perthiyl radical, observed by EPR spectroscopy, is due to the homolytic cleavage of the 2′-carbon−sulfur bond.

tion of R2. On the other hand, the perthiyl radical, or a derived species, does not lead to R1 inactivation, suggesting that, under assay conditions, i.e. in the presence of DTT and thioredoxin, active site-reduced cysteines are readily regenerated from modified cysteines as they are from cysteines oxidized by substrates. DTT might also protect protein R1 from inactivation by the 3′-keto derivative (Harris et al., 1984).

The model shown in Scheme 3 is the minimal one that fits to the experimental data. First, formation of the perthiyl radical requires oxygen to generate a S−S bond. In the absence of oxygen, no disulfide is formed and compound **1** is likely to behave as a substrate, explaining why no loss of the tyrosyl radical could be observed. This needs to be shown experimentally. Second, the formation of the perthiyl radical requires that the active site cysteines of R1 are in the reduced form. If they are oxidized or changed to serines, the tyrosyl radical disappears as expected, but no perthiyl could be observed. It has been previously reported that, also with natural substrates, mutant R1s induce a slow irreversible loss of the R2 radical.

In summary, we have shown that 2′-deoxy-2′-mercapto ribonucleotide is a remarkable inhibitor of RDPR. Only the small subunit of the enzyme is inactivated, with its tyrosyl radical changed into a normal tyrosine. Transfer of the radical to another site has been demonstrated by direct detection of a protein R1-based RSS• radical. During this work, a study on pyruvate formate-lyase was reported, which also led to the observation of a transient perthiyl radical (Parast et al., 1995). Moreover, this is the second report of such a radical transfer from the small to the large subunit of RDPR, mediated by a mechanism-based inhibitor. The first one led to the observation of the RSN• radical located on cysteine 225 of R1 generated during R2 tyrosyl radical scavenging by 2′-azido-2′-deoxyuridine 5′-diphosphate (van der Donk et al., 1995). Further studies are required to understand the mechanism of the reaction, with special focus on the identification of products of decomposition of the substrate analog.

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