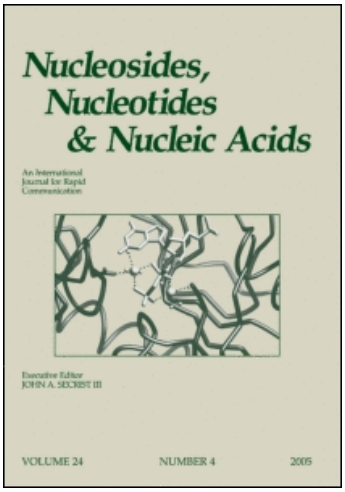

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Ribonucleotide Reductases: Radical Chemistry and Inhibition at the Active Site

Morris J. Robins[a]
[a] Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah, USA

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Ribonucleotide Reductases: Radical Chemistry and Inhibition at the Active Site

Morris J. Robins*

Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah, USA

ABSTRACT

Ribonucleoside 5′-diphosphate reductases (RDPRs) have been studied for several decades. Increasingly sophisticated mechanisms have been proposed for the reduction of natural substrate ribonucleotides to their 2′-deoxy counterparts and for mechanism-based inactivation of RDPRs with 2′-substituted-ribonucleotides. We now discuss biomimetic reactions of model substrate and inhibitor analogues, which clarify three aspects of previously proposed mechanisms postulated to occur at the active site of RDPRs.

Key Words: Ribonucleotide reductase; Ribonucleoside 5′-diphosphate reductase (RDPR); Reduction of ribonucleotides to 2′-deoxynucleotides; Mechanism-based inactivation of RDPR; Fenton chemistry; Free radical chemistry; Anion vs. radical elimination; Biomimetic reaction models; Thiohemiacetal formation.

BACKGROUND AND INTRODUCTION

Ribonucleotide reductases (RNRs) are ubiquitous enzymes that perform the only *de novo* biosynthesis of 2′-deoxynucleotide building blocks for DNA. A number

*Correspondence: Morris J. Robins, Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah 84602-5700, USA; E-mail: morris-robins@byu.edu.

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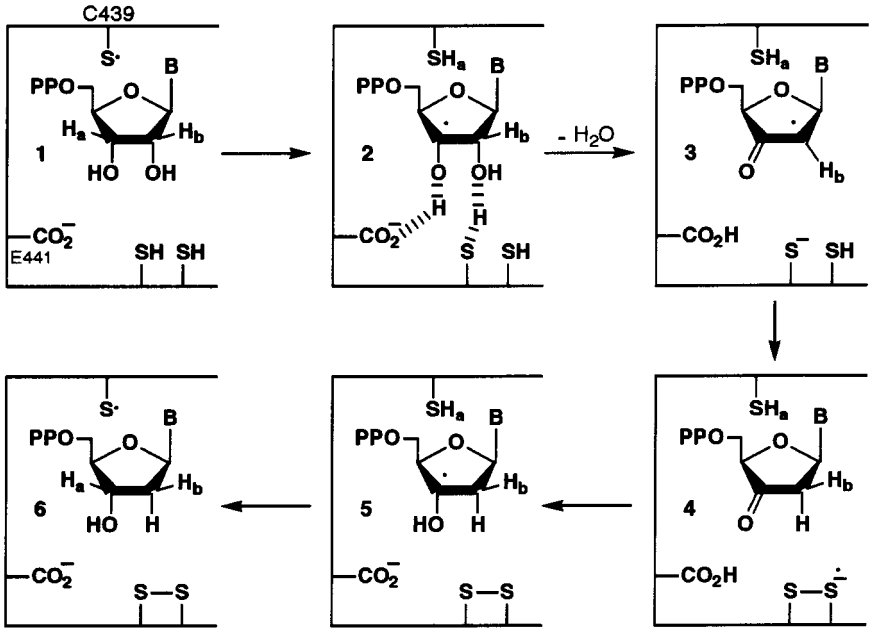
of reviews are available,[1–5] and the present treatment is focused on mechanisms of substrate reduction by class I enzymes [such as the ribonucleoside diphosphate reductases (RDPRs) from *Escherichia coli* and mammalian cells] and mechanism-based inactivation by substrate analogues. The class I enzymes have dimeric subunit structures of the $\alpha_2\beta_2$ type. The R1 subunit contains redox-active cysteine residues, binding sites for the nucleoside diphosphate (NDP) substrates, and sites for binding nucleoside triphosphates for allosteric control of reaction rates and substrate specificities. The R2 subunit contains a di-iron complex and a tyrosyl radical, which function as a long-range radical-transfer initiation system for the reduction chemistry. Fascinating mechanistic sequences have been elucidated, primarily by the elegant biochemical and chemical studies of the Swedish[3] and Stubbe[4] groups.

Stubbe and coworkers performed extensive chemical/enzymatic studies with labeled and substituted nucleoside analogues, and proposed increasingly sophisticated mechanistic models.[4] These working hypotheses were based on analogy with free radical chemistry of Fenton reagents studied by Walling[6] and others.[7] Fenton chemistry [hydrogen peroxide and an iron(II) salt in acidic aqueous solution] with ethylene glycol involves abstraction of a hydrogen atom from carbon by a hydroxyl radical, protonation of the vicinal hydroxyl group, and departure of H_2O. Proton loss from the hydroxyl group of the resulting resonance-stabilized radical cation and reduction by Fe[II] gives ethanal. Stubbe proposed[4c,d,8] that long-range electron transfer from Cys439 in the R1 subunit to a tyrosyl radical in R2 of RDPR from *E. coli* generates a proximal thiyl radical, which abstracts hydrogen from C3′ of the NDP substrate in **1** (Sch. 1) to give the C3′ radical in **2**.



Scheme 1. Stubbe mechanism for reduction of NDP substrates by RDPR.[4c]

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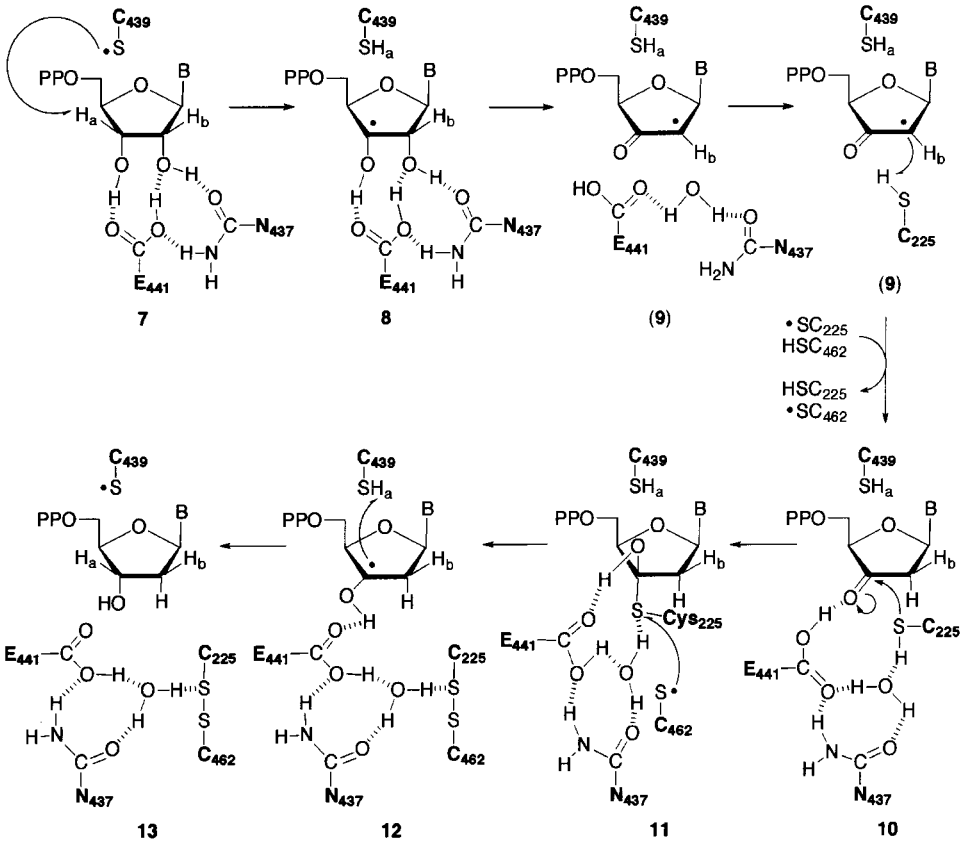
Hydrogen bonding from a Cys thiol at the α-face of the furanose ring to the 2′-hydroxyl group promotes departure of H_2O from C2′, and transfer of the 3′-hydroxyl proton to Glu441 gives the C2′ radical in **3**. Abstraction of hydrogen from a second Cys thiol at the α-face gives the 2′-deoxy-3′-oxonucleotide in **4** and a disulfide radical anion. Electron transfer from the radical anion to the ketone and protonation of O3′ by Glu441 was postulated to give the C3′ radical in **5**. Hydrogen transfer to C3′ from Cys439 completes reduction to the 2′-deoxynucleotide (dNDP) in **6** and regeneration of the Cys439 thiyl radical. Redox reactions that involve several enzymes and cofactors convert the cysteine disulfide into a Cys pair for the next catalytic cycle.[1,3]

X-ray crystal structures of the R1 and R2 subunits of RDPR have been determined,[9] and an extensive review of structure and function is available.[3f] Siegbahn reinvestigated the Stubbe mechanism with theoretical calculations[10] that employed amino acid residues at the presumed active site in the X-ray structures. This modified mechanism (Sch. 2) incorporates the fundamentals of Stubbe's hypothesis, but differs in important aspects. His calculations support abstraction of H3′ from the NDP substrate by the Cys439 thiyl radical in the H-bonded matrix in **7**. Hydrogen-bonding



Scheme 2. Siegbahn machanism for reduction of NDP substrates by RDPR.[10]

with the C3′ radical in **8** promotes deprotonation of O3′ (Glu441) and loss of O2′ (as H_2O, H-bonded with Asn437 and Glu441) to give the 3′-oxo radical in **9**. Hydrogen transfer from Cys225 in **9** gives the 2′-deoxy-3′-ketone in **10**, and thermoneutral hydrogen transfer from Cys462 to the thiyl radical of Cys225 produces the nucleophile for the next step. The 2′-deoxy-3′-oxonucleotide in **10** is by far the most stable intermediate in the sequence,[10] and we have prepared and characterized analogous 2′-deoxy-3′-oxonucleoside derivatives.[11] Therefore, direct reduction of the ketone group in **10** would have a high energy barrier. Calculations[10] were incompatible with several pathways for electron transfer from a sulfur radical anion[4] to the 3′-ketone. A chemically plausible pathway (with appropriate energy barriers[10] for observed enzyme kinetics) involves general-acid protonation of O3′ in **10** by Glu441. Hydrogen-bonding of the thiol proton, and nucleophilic attack of Cys225 at C3′ produces the thiohemiacetal in **11**. Attack of the proximal Cys462 thiyl radical at Cys225 gives the Cys225–Cys462 disulfide and the C3′ radical in **12**. Abstraction of hydrogen from Cys439 by C3′ in **12** regenerates the Cys439 thiyl radical and completes the reduction cycle from NDP in **7** to dNDP in **13**. This hypothesis retains the radical chemistry proposed by Stubbe, but has two significant modifications:

(i) Protonation of O2′ and loss of H_2O was calculated to be more favorable in the protein-bound matricies in **7**–**9** (Sch. 2) than with Cys (Sch. 1).
(ii) Acid-catalyzed nucleophilic addition of Cys225 to the 3′-ketone gives a chemically plausible conversion to the thiohemiacetal[10] in **11**.

Because RNRs provide the only known *de novo* pathway to dNTP precursors for DNA biosynthesis, inhibition of these enzymes provides a rational approach for discovery of drugs against uncontrolled cellular proliferation and virus replication. Thelander et al.[12] first studied mechanism-based inactivation of RDPR with the 5′-diphosphates of 2′-azido-2′-deoxycytidine and 2′-chloro-2′-deoxy(cytidine and uridine). Stubbe and coworkers postulated mechanisms based on characterization of products from incubation of labeled and substituted nucleotide derivatives with RDPR. Her mechanism for inactivation of RDPR with 2′-chloro-dNDPs in **14** (Sch. 3)[4c] begins with Cys439 thiyl radical abstraction of hydrogen from C3′. By analogy with Fenton chemistry (with 2-chloroethanol[6a]), she proposed spontaneous loss of chloride from C2′ in **15**,[4a] with accompanying general-base deprotonation of O3′ by Glu441,[4c] to give the same 3′-oxo C2′ radical in **16** that is produced in substrate reductions. She reasoned that differences in pathways to observed products resulted from the different oxidation states of Cys225/Cys462 (Sch. 3) vs. the radical anion (Sch. 1). Hydrogen transfer in **16** from Cys 225/462 at the α-face of C2′, or Cys439 at the β-face, gives the 2′-deoxy-3′-oxonucleotide in **17** (or with a different hydrogen isotope at C2′ from ^3H_a of a labeled substrate in **17′**). Absence of the radical anion results in dissociation from the active site without reduction of the ketone in **17**/**17′**. The reactive Michael acceptor **18** is formed by β-eliminations of H2′ and nucleobase (from C1′) and H4′ and pyrophosphate (from C5′). Michael alkylations of thiol and/or nitrogen residues in the enzyme result in mechanism-based inactivation of RDPR.

Such suicide inactivation of RDPR has been demonstrated with other 2′-substituted-dNDPs,[4a,c] and 2′-deoxy-2′,2′-difluorocytidine[13] (F_2dCyd, gemcitabine) is an

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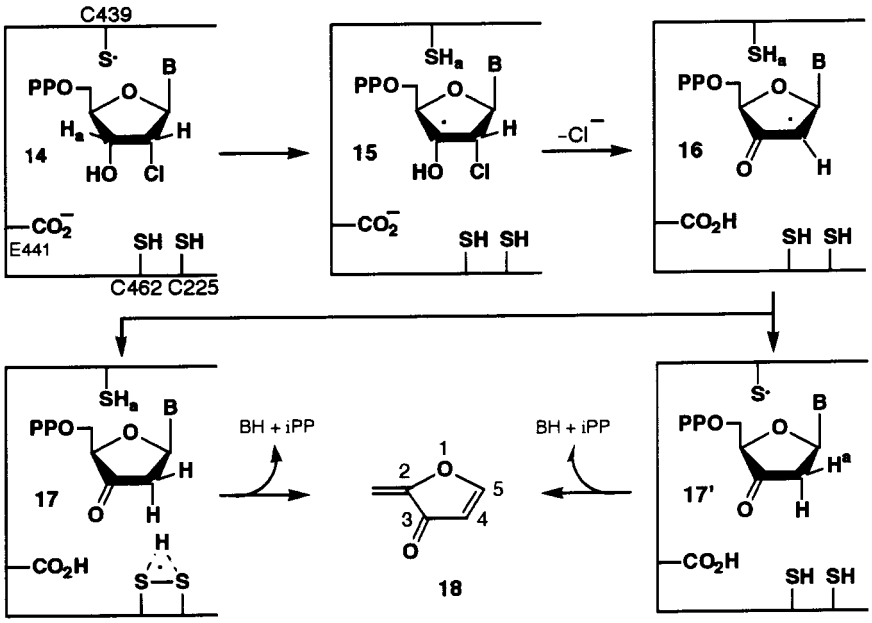
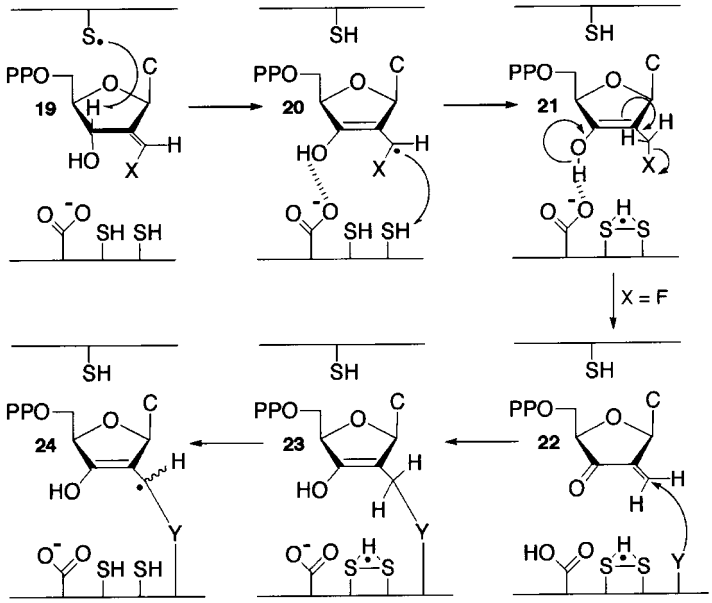


Scheme 3. Stubbe mechanism for inactivation of RDPR with CldNDPs.[4c]

effective drug for treatment of several human tumors.[14] The major mode of action of gemcitabine involves incorporation of its triphosphate into DNA,[14a,15] and the diphosphate is an extremely potent mechanism-based inactivator of RDPR[16] (which causes self-potentiation of incorporation as a consequence of diminished dNTP pools). Incubation of F_2dCDP with RDPR causes rapid and stoichiometric inactivation of the enzyme,[16] and cleavage of the inhibitor occurs to give cytosine, two equivalents of fluoride, and a new radical species.[16b] The rapid and stoichiometric inactivation makes mechanistic studies with the enzyme more difficult. Rational design of new mechanism-based inhibitors of RDPR necessarily depends on elucidation of the most accurate approximation of the enzyme mechanism, and biomimetic simulations with appropriate substrate analogues and reaction conditions provide a valuable approach for further investigations.

Ueda, Matsuda, and coworkers[17] prepared 2′-deoxy-2′-methylenenucleosides, and reasoned that incorporation of MdNTPs into DNA would give unstable 3′-phosphodiester linkages (DNA cleavage) via dissociation of the phosphate from C3′ to generate an allylic cation. We[18] synthesized methylene analogues as prodrugs of 5′-diphosphate inactivators of RDPR, because abstraction of H3′ from MdNDPs would give allylic radicals. Inhibition of RDPR by MdCDP was demonstrated,[16a] and the cytotoxic potency and anticancer efficacy of MdCyd were studied.[17,18b] McCarthy and coworkers extended this concept with (*E*)-2′-deoxy-2′-(fluoromethylene)cytidine[19] (FMdCyd). FMdCDP is a potent inactivator of RDPR,[20] and FMdCTP also is incorporated into DNA.[21] Thus, such deoxynucleoside prodrugs are converted into 5′-(mono, di, and tri)phosphates, and cytotoxic effects result from



Scheme 4. Mechanism for inactivation of RDPR with MdCDP[16a] or FMdCDP.[20]

incorporation of the triphosphates into DNA.[15,21] Inactivation of RDPR by the diphosphates diminishes natural dNTP pools, which self-potentiates enhanced incorporation of the dCTP analogues.

Scheme 4 illustrates inactivation of RDPR by MdCDP[16a] or FMdCDP.[20] Abstraction of hydrogen from C3′ in **19** by the Cys439 thiyl radical produces the allylic radical in **20**. Hydrogen transfer from a residue in the enzyme to the exocyclic terminus of the radical gives the methyl or fluoromethyl enols in **21** [or tautomeric α-(methyl or fluoromethyl) ketones]. Conjugate (enol) or β-elimination (ketone) of a proton and nucleobase, and H4′ and pyrophosphate, generates 4-methyl-2(*H*)-methylenefuran-3-one (4-methyl-**18**, Sch. 3) from MdCDP, and Michael alkylation of the enzyme causes inactivation.[16a] The intermediate in **21** (X = F) from FMdCDP could follow a parallel sequence, but additional chemistry is possible. Conjugate elimination of fluoride from the enol in **21** (X = F) gives the enone in **22** in proximity with a nucleophilic residue. Michael addition in **23** and hydrogen/proton transfer steps produce a persistent radical whose EPR spectra were consistent with the structure in **24**.[20] The *Z*-diastereomer of FMdCyd (methylene F and H interchanged) had insignificant biological activity. However, chemical phosphorylation gave the (*Z*)-5′-diphosphate, which also was a potent inactivator of RDPR.[22] It is important to remember that most nucleosides are *prodrugs* of biologically active phosphates, and that observed lack of activity might result from failure to function as a kinase substrate rather than failure to bind/react at the ultimate nucleotide target.

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We prepared 2′-deoxy-2′-spirocyclopropane derivatives of nucleosides (and their di- and triphosphates) as a new type of mechanism-based inactivator of RNRs.[23] It is well-known that spirocyclopropylcarbinyl radicals undergo ring opening at high rates to give radical-substituted alkenes.[24] Model reactions that generated a C3′ radical with 2′-deoxy-5′-*O*-*tert*-butyldimethylsilyl-2′-spirocyclopropyladenosine (5′-*O*-TBDMS-cPdAdo) or 5′-*O*-TBDMS-cPdUrd proceeded with cleavage of the cyclopropane ring to give products derived from ethyl radical formation.[23] However, the nucleoside analogues did not have significant activity against cancer cells or viruses. The cPdCDP analogue was a slow and inefficient inactivator of RDPR, an enzyme with stringent spatial tolerance in the region "above" where C2′/C3′ of the furanose ring is bound.[25] RTPR from *Lactobacillus leichmannii* has greater spatial tolerance for substituents at the β-face,[26] and cPdCTP caused rapid inactivation of that enzyme.[25] Matsuda and coworkers prepared 3′-*C*-ethynyl analogues of ribonucleosides, and some of these compounds have good anticancer activity.[27] One design rationale for those derivatives was to trap RDPR radical species with an alkyne at C3′,[27a] but no inactivation of the enzyme was observed. This also is consistent with the stringent spatial requirements "above" C3′.

The Stubbe mechanisms[4c,d] for reduction of NDPs by RDPR and inactivation of the enzyme by CldNDPs are generally accepted. However, three concerns had not been addressed in a rigorous manner. The first is that abstraction of hydrogen from C3′ by a thiyl radical on Cys439 might be contrathermodynamic.[4d] Calculations[10] indicate that coupling of this step with sequential reactions provides viable thermodynamic and activation parameters. However, model reactions invoked[4] to support abstraction of hydrogen by thiyl radicals utilized radiolysis of aqueous solutions, and were not closely related to processes postulated to occur at the active site of RNRs. Lenz and Giese[28] prepared 3′-*C*-(phenylselenylcarbonyl) nucleoside analogues and subjected these selenoesters to photolysis in polar solutions. Formation of C3′ radicals and loss of the 2′-hydroxyl group gave products consistent with intermediate steps of the Stubbe mechanism. They[28] also observed general-base catalysis (with a carboxylate salt) of the loss of O2′ in harmony with X-ray structures and calculations of H-bonding of Glu441 with the 3′-hydroxyl of substrate NDPs. However, reactions with high energy input (radiolysis or photolysis) in solutions of high dielectric constant are dissimilar to the environment at the active site of RDPR (calculated[10] dielectric constant ~4). Also, Cai and Roberts[29] treated a chiral tetrahydrofuran derivative with thiyl radicals and observed varying degrees of racemization (abstraction and return of hydrogen at a carbon stereocenter with an oxygen substituent). Highly electrophilic thiyl radicals promoted racemization, whereas nucleophilic alkane-thiyl radicals were poor hydrogen-transfer species. In fact, a thiyl radical derived from the methyl ester of cysteine did not execute *any* hydrogen transfer.[29] Such abstraction of hydrogen from C3′ of NDPs by the Cys439 thiyl radical is the key initiation step for RNR-mediated radical cascades with both substrates and inactivators.[4c,d,10]

The second concern is the nature of species that depart from C2′, especially in the mechanism for inactivation of RDPR with CldNDPs and analogues. Fenton chemistry with 2-chloroethanol was performed in aqueous solution.[6a] Spontaneous loss of chloride and formation of a resonance-stabilized radical cation is plausible in

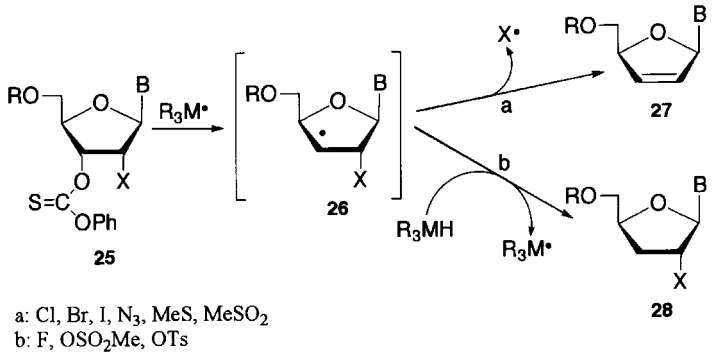
that medium, but not at C2′ of a nucleotide in the active site (dielectric constant ~4) of RDPR. The anomeric carbon (C1′) of nucleotides is highly electron deficient, and this dramatically raises the energy of intermediates with positive charge character at C2′.[30] A concerted processes or departure of a radical rather than an anionic species from C2′ is strongly favored. Furthermore, Wagner has demonstrated facile radical β-elimination processes that result from abstraction of an α-hydrogen from β-substituted [chloro, bromo, iodo, and (alkyl or aryl) thio] compounds.[31]

The third concern is the electron-transfer step in the Stubbe hypothesis[4c] for reduction of the 2′-deoxy-3′-oxo intermediate in **4** (Sch. 1). Siegbahn found no electron-transfer process that was energetically feasible.[10] However, calculations revealed a chemically plausible process **10** → **12** (Sch. 2) via general acid-catalyzed formation of a thiohemiacetal and displacement of a C3′ radical from a thioether by a cysteine radical. We have observed spontaneous formation of an analogous thiohemiacetal in a furan model, and have performed other experiments that clarify and illuminate aspects of the three mechanistic concerns.

RESULTS AND DISCUSSION

We initially addressed the second concern, that β-elimination of radicals from C2′ upon generation of a radical at C3′ should be more energetically favorable than elimination of anions vicinal to the electron-deficient anomeric center, by treatment of 5′-*O*-TBDMS-3′-*O*-phenoxythiocarbonyl-2′-(substituted)nucleosides **25** (Sch. 5) with tributylstannane/AIBN or triphenylsilane/dibenzoyl peroxide in hot toluene.[32] Attack of stannyl or silyl radicals on the PTC ester and β-scission gives a C3′ radical **26**. Potential avenues for **26** include:

(i) β-elimination of a radical from C2′ with formation of a 2′,3′ π-bond,
(ii) β-elimination of an anion from C2′ with formation of a cation-radical, which could undergo further reduction and/or coupling with a nucleophile, or
(iii) Hydrogen transfer directly from tin or silicon to give a 3′-deoxy compound.



Scheme 5. Radical elimination vs. hydrogen transfer with 2′-substituted 3′-radicals.[32]

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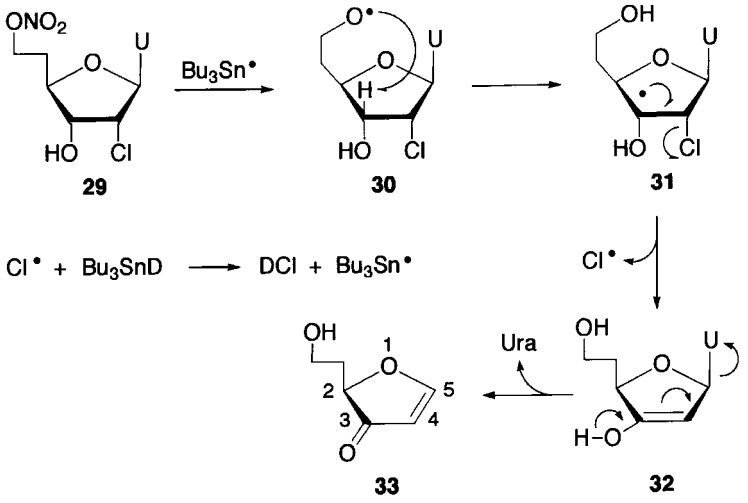
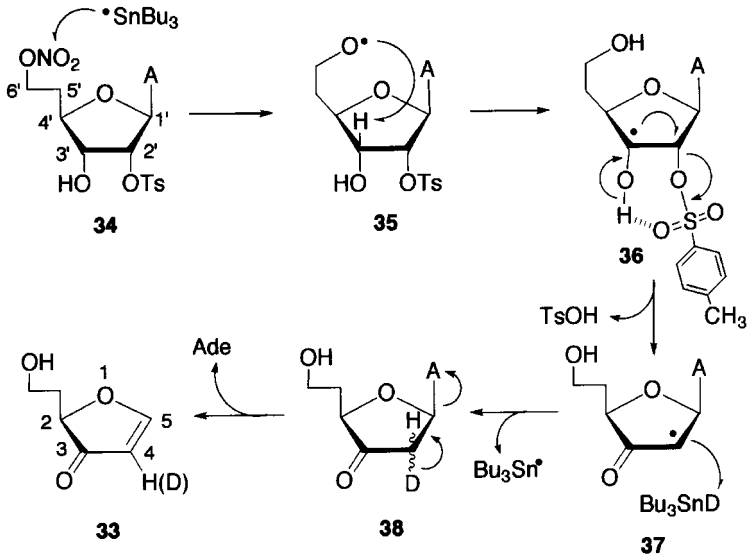
Analogues with X = azido, bromo, chloro, iodo, methylthio, or methylsulfonyl underwent β-elimination of a radical to give a 2′,3′-alkene **27**.[32] By contrast, analogues with X = fluoro, methanesulfonate, or *p*-toluenesulfonate underwent direct hydrogen transfer to C3′ to give a 5′-*O*-TBDMS-3′-deoxy-2′-substituted derivative **28**. These experiments demonstrate that β-elimination of radicals is clearly favored over anions, because mesylate and tosylate anions are better leaving groups than chloride. Moreover, the high energy content of fluorine, mesyloxy, and tosyloxy radicals precludes homolytic cleavage of these C–F and C–O bonds. The results are definitive for such β-eliminations with a secondary alkyl radical center at C3′.[32] However, the initial NDP radicals generated with RDPR are stabilized by a 3′-hydroxyl substituent. It was known that 1,5-hydrogen transfer from C–H to an oxy radical was favored over transfers with 5- or 7-membered transition states,[31,33] and that treatment of nitrate esters with tributylstannane produced oxy radicals.[34] Therefore, we selected 5′-deoxy-6′-*O*-nitro-2′-(substituted) homonucleoside derivatives for our biomimetic studies of processes postulated to occur at the active site of RDPR.

Glucose was converted into 5-deoxy-β-D-*ribo*-hexofuranosyl (homoribose) derivatives, and Vorbrüggen coupling of sugar derivatives gave the homo(adenosine and uridine) analogues.[35] Treatment of 6′-*O*-nitro esters of homonucleosides with Bu_3SnH/AIBN generated 6′-oxy radicals that executed 1,5-hydrogen abstraction to give C3′ radicals, and deuterium transfer to C3′ with Bu_3SnD verified the radical-relay from O6′.[35a,c] Known transformations gave 2′-chloro-2′-deoxy-6′-*O*-nitrohomouridine[35a,c] (**29**) (Sch. 6) and 6′-*O*-nitro-2′-*O*-tosylhomoadenosine[35b,c] (**34**) (Sch. 7) for biomimetic experiments. The stable 2′-*O*-tosylhomoadenosine derivative **34** was required, because an analogous 2′-*O*-tosylhomouridine underwent intramolecular cyclization to give 2,2′-anhydro cyclonucleoside byproducts under the thermal conditions[34] we employed for generation of 6′-oxy radicals from 6′-*O*-nitro esters.



Scheme 6. Radical elimination of Cl^- with a 2′-chloro-2′-deoxynucleoside model.[35c]



Scheme 7. Concerted elimination of HOTs with a 2′-*O*-tosylnucleoside model.[35c]

We were gratified that treatment of **29** with Bu_3SnH/AIBN/benzene/Δ resulted in formation of 2-(2-hydroxyethyl)-3(2*H*)-furanone (**33**) in harmony with our mechanistic predictions.[35a,c] Treatment of **29** with Bu_3SnD under identical conditions gave furanone **33** with no detected incorporation of deuterium (MS and ^1H NMR). This is consistent with loss of a chlorine atom rather than a chloride anion from radical **31**. Abstraction of nitro from **29** by a stannyl radical generates the 6′-oxy radical **30**. A 1,5-hydrogen transfer from C3′ to O6′ gives **31**, and departure of chlorine from **31** gives the enol **32**. Conjugate elimination of the hydroxyl proton and uracil from **32** (or tautomerization and β-elimination) completes the pathway to furanone **33**. The chlorine atom released by **31** abstracts hydrogen (or deuterium) from the stannane to propagate the radical chain reaction. This type of radical elimination process would be viable for leaving groups, X, at C2′ with appropriate C–X bond dissociation energies, but not for those with high BDEs (e.g., C–F or C–OTs).

Treatment of the 2′-*O*-tosyl derivative **34** under the same conditions gave the identical 2-(2-hydroxyethyl)-3(2*H*)-furanone (**33**) (Sch. 7). Repetition of this reaction with Bu_3SnD gave **33** with ~30% incorporation of deuterium at C4 (corresponding to C2′ in substrate **34**) in complete agreement with our mechanistic predictions.[35b,c] Abstraction of nitro from **34** by a stannyl radical and 1,5-hydrogen transfer from C3′ to O6′ in **35** proceed the same (Sch. 7) as with the 2′-chloro analogue (**29** → **31**, Sch. 6). However, homolytic cleavage of the C–OTs bond is energetically prohibitive. Therefore, concerted loss of the 3′-hydroxyl proton and OTs from C2′ generates the delocalized C2′ radical **37** (via an energetically favorable[36] 7-membered transition state in **36**, without localization of unfavorable positive charge at C2′). Hydrogen (deuterium) transfer from the stannane occurs

preferentially from the less-hindered α-face at C2′ of **37** to give the 2′-deoxy-3′-oxo-homonucleoside **38**.

We have previously shown that deuterium transfer from Bu_3SnD to ribonucleoside-derived C2′ radicals occurs at the α-face with ~85% diastereoselectivity.[37] Spontaneous β-elimination of H2′ and the nucleobase proceeds readily with 2′-deoxy-3′-oxonucleoside derivatives,[11] and 1,2-anti eliminations are energetically favored. Anti-elimination of the proton/deutron (~3:7) at the α-face of C2′ and the nucleobase at C1′ in **38** would give furanone **33** with ~30% retention of deuterium, as observed. [A control reaction[35b,c] with a 2′-deoxy-3′-oxonucleoside derived from adenosine (~85% ^2H at the α-face of C2′) gave a furanone with ~15% deuterium retention.]

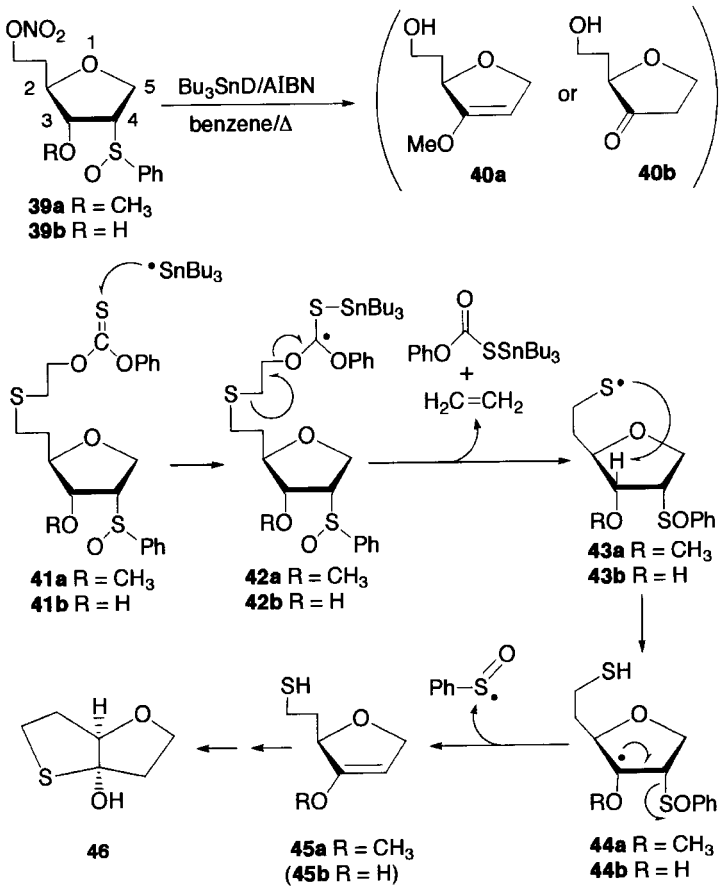
These experiments clearly illustrate similarities and differences between mechanisms for the reduction of NDP substrates by RDPR and inactivation of the enzyme by 2′-X-NDP analogues. NDP substrates with oxygen (or inactivators with fluorine) at C2′ rely on the H-bonded protein matrix for abstraction of the 3′-hydroxyl proton in concert with departure of H-bonded hydroxyl (or fluoride) from C2′ to produce a delocalized 3′-oxo-2′-radical intermediate. BDEs of C–O (and C–F) bonds preclude spontaneous radical elimination, and departure of anionic species with concomitant generation of positive charge at C2′ is energetically prohibitive.[30] By contrast, inactivators with substituents at C2′ that have appropriate BDEs (e.g., C–Cl, C–Br, $C–N_3$, C–S) can undergo spontaneous radical β-elimination upon formation of the initial C3′ radical. Thus, substrate-derived C2′ radicals undergo hydrogen transfer from Cys225 to give the 2′-deoxy-3′-oxo intermediate in **10** (Sch. 2). The resulting Cys225 radical undergoes hydrogen transfer with Cys462, and then participates in general acid-catalyzed thiohemiacetal formation at C3′. Displacement of Cys225 with the Cys462 thiyl radical gives a cysteine disulfide and the C3′ radical, which undergoes hydrogen transfer with Cys439 to produce dNDP product and regenerate the Cys439 radical.

Partitioning between reduction to dNDP product and inactivation of RNRs can occur with 2′-fluoro, and to a lesser extent with 2′-chloro, nucleotide analogues.[4] These results also are accommodated by our mechanistic hypotheses. Abstraction of H3′ by the Cys439 radical generates a C3′ radical with a 2′-substituent, and H-bonding to fluorine is strong. Concerted loss of H-bonded HF and alternative substrate processing would give the dNDP product. Because HF forms even stronger H-bonds than H_2O, formation of a 2′-deoxy-3′-oxonucleotide could proceed, but HF might interfere with subsequent reduction steps. Dissociation from the active site and β-eliminations generate the inactivating furanone **18** (Sch. 3). Generation of the C3′ radical with a 2′-chloro substituent results in β-elimination of a chlorine atom in proximity with Cys225. Hydrogen transfer from Cys225 to chlorine would produce the Cys225 thiyl radical and HCl adjacent to C2′. Conjugate elimination of the 3′-hydroxyl proton and nucleobase generates an enone, which would dissociate to form the Michael inactivator **18**. Alternatively, proton transfers from the 3′-hydroxyl to Glu441 and HCl to C2′ would give the intermediate 2′-deoxy-3′-oxonucleotide in **10**. Only the presence of chloride and the absence of H-bonded water would be different than the usual substrate reduction (Sch. 2). Therefore, alternative substrate reduction (dNDP plus chloride) and inactivation (conjugate elimination to give furanone, after chlorine atom loss)

are competing processes at the active site. These hypotheses rationalize the second concern with the Stubbe mechanisms.

The first concern was the thermodynamic validity of abstraction of H3′ by the nucleophilic Cys439 thiyl radical, especially since no abstraction of hydrogen from an α-oxy ring carbon by a cysteine ester thiyl radical was observed by Cai and Roberts.[29] Our 6′-*O*-nitrohomonucleoside studies clearly demonstrated that an oxy radical can execute 1,5-hydrogen transfer from C3′. Our first attempts to probe analogous transfer to a thiyl radical were performed on a more synthetically accessible glucofuranose platform.[38] However, generation of analogous oxygen, nitrogen, and sulfur radicals gave deuterium-transfer incorporation only with the oxygen and nitrogen radicals.

We then prepared a more conformationally mobile tetrahydrofuran system derived from glucose,[39] and performed control reactions with an oxy-radical relay. Treatment of the 3-methoxy-2-(2-*O*-nitroethyloxy)-4-(phenylsulfinyl)tetrahydrofuran derivative **39a** (D-ribo stereochemistry) (Sch. 8) with Bu_3SnD/AIBN/



Scheme 8. H3′ abstraction by a thiyl radical[39] and generation of thiohemiacetal.

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benzene/Δ resulted in generation of the oxy radical, 1,5-hydrogen transfer from C3, and β-elimination of the phenylsulfinyl radical to give enol ether **40a**.[39] Parallel treatment of the analogous 3-hydroxy compound **39b** gave the stable 3-keto tautomer **40b**. Uncertainty regarding possible failure to produce thiyl radicals was addressed by relay generation with a 2-{2-[(phenoxythiocarbonyl)oxy]ethylthio} moiety on the 2-ethyl group. Attack of a stannyl radical at thiocarbonyl sulfur followed by β-scission generates a transient ethylthioethyl radical, which undergoes β-scission with loss of ethene (in **42**) to produce thiyl radicals **43** of defined integrity. Syringe-pump addition of Bu_3SnD/AIBN to the 3-methoxy derivative **41a**/benzene/Δ resulted in formation of the enol-ether thiol **45a**.[39] Generation of **45a** firmly establishes that a nucleophilic thiyl radical in appropriate proximity can execute 1,5-hydrogen transfer (**43a** → **44a**) from a secondary carbon with an oxygen substituent. Parallel treatment of the 3-hydroxy analogue **41b** produced the thiohemiacetal **46**. This result corroborated 1,5-hydrogen transfer from C3 to a nucleophilic thiyl radical (**43b** → **44b**), the first concern with the Stubbe/Siegbahn mechanism—and also demonstrated the facility with which addition of an alkylthiol to the 3-oxo function occurs. Loss of a phenylsulfinyl radical from **44b** generates enol **45b**, which tautomerizes to give the 3-oxo compound analogous to **40b**. However, spontaneous intramolecular addition of the thiol to the 3-ketone gives thiohemiacetal **46**, the stable isolated product. This is in contrast with the alcohol **40b**, which was isolated as the stable ketone.

General acid-catalyzed addition of Cys225 to the carbonyl group of 2′-deoxy-3′-oxonucleotides is a new feature of Siegbahn's hypothesis for conversion of the stable intermediate in **10** (Sch. 2) into product dNDPs. Our observation of the *spontaneous* cyclization to form **46** (Sch. 8) in a solvent with a dielectric constant (2.3) similar to that estimated for the active site of RDPR (∼4) provides a firm chemical basis for that pathway. Thus, biomimetic studies with generation of nucleophilic alkyl-thiyl radicals[39] have placed hydrogen abstraction from C3′ by the Cys439 thiyl radical *and* the pathway for reduction of the 3′-oxo group via addition of Cys225 to give a thiohemiacetal intermediate on a secure foundation.

SUMMARY AND CONCLUSIONS

We have studied chemically simplified nucleoside mimics, which undergo radical-induced reactions that are analogous to those postulated to occur at active sites of RNRs. We have demonstrated that certain radical elimination processes are more plausible than anion elimination mechanisms that were previously postulated by analogy with Fenton chemistry in aqueous solutions. We have demonstrated the chemical feasibility of thiyl radical abstraction of hydrogen from C3′ of nucleoside models, and observed spontaneous formation of a stable thiohemiacetal that is analogous to a key intermediate in a plausible new pathway for reduction of 2′-deoxy-3′-oxonucleotides to give product dNDPs. Our biomimetic reactions provide a firm basis for the design and investigation of new mechanism-based inhibitors of RNRs. Definitive rationalization of the mechanism of inactivation of RNRs by gemcitabine phosphates might yield to this approach. Discovery of new inactivators of RNRs with applications to human disease remains the ultimate goal of our efforts in this area.

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