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Reactivity of the Major Product of C5'-Oxidative DNA Damage in Nucleosome Core Particles

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Abstract

The major pathway for DNA damage following hydrogen atom abstraction from the C5'-position results in direct strand scission and concomitant formation of a 5'-aldehyde containing nucleotide (e.g. T-al). We determined that the half-life for alkali-labile T-al in free DNA under physiological conditions varies from 5 – 12 days. T-al reactivity was examined at 3 positions within nucleosome core particles (NCPs). β-Elimination increased >2.5-fold when T-al was proximal to the lysine rich histone H4 tail. No difference in reactivity between free DNA and NCPs was observed when T-al was distal from the histone tails. Position dependent involvement of histone tails in T-al elimination was gleaned from experiments using sodium cyanoborohydride and histone protein variants. The enhancement of T-al elimination in NCPs is significantly smaller than previously observed for abasic sites. Computational studies comparing elimination from T-al and abasic sites indicate that the barrier for the rate determining step in the latter is 2.6 kcal/mol lower in energy and is stabilized by a hydrogen bond between C4-hydroxyl group and phosphate leaving group. The long lifetime for T-al in NCPs, combined with what is known about its repair suggests that this DNA lesion may pose significant challenges within cells.

Graphical Abstract

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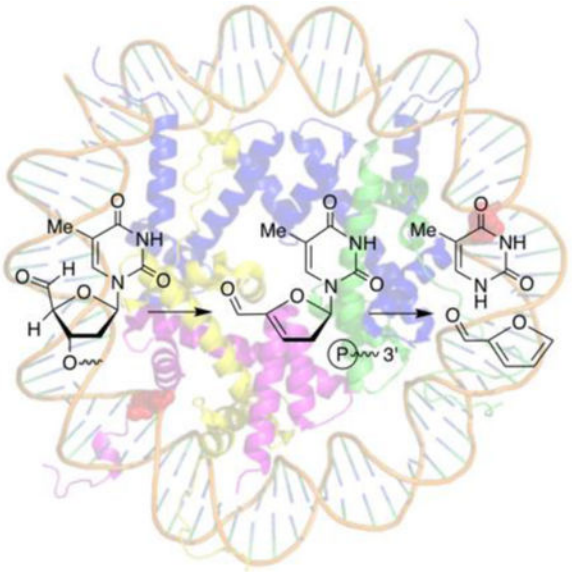

C5'-Aldehydes (e.g. T-al) are the major products of C5'-oxidation in DNA. T-al undergoes β-elimination and the process is catalyzed by the lysine rich N-terminal tail of histones. However, the rate acceleration of this process is modest in nucleosome core particles. Hence, C5'-aldehydes such as T-al are expected to have long lifetimes in nuclear DNA and may affect biochemical processes in cells.

The C5'-hydrogen atoms of nucleotides in DNA are highly accessible to diffusible species and the respective carbon-hydrogen bonds are weaker than most other such bonds in nucleotides.[1] Consequently, the C5'-hydrogens are believed to be the most frequently abstracted by hydroxyl radical.[2] A variety of minor groove binding antitumor agents and other DNA damaging agents also abstract a C5'-hydrogen atom (C5'•, Scheme 1) resulting in direct strand scission.[3] Mutagenic cyclonucleotides (e.g. cdG) that are resistant to base excision repair are also produced from purines.[4] A 5'-aldehyde containing nucleotide (e.g. T-al) or an oxidized abasic site (DOB) is formed concomitantly with strand scission. Although DOB is a minor product, it has significant biochemical effects. For instance, the oxidized abasic site is a potent irreversible inhibitor of DNA polymerases and gives rise to histone lysine modification in nucleosome core particles (NCPs).[5] T-al, the most well characterized C5'-nucleotide aldehyde, is resistant to short patch base excision repair (BER).[6] T-al is excised by the long patch BER pathway that utilizes DNA polymerase β and flap endonuclease 1, as well as nucleotide excision repair, albeit less efficiently than other DNA lesions. T-al is also an alkali-labile lesion (Scheme 1) whose reactivity may be affected by Schiff base formation.[3b, 7] These properties led us to address whether histone proteins within NCPs catalyze β-elimination from T-al via transient DNA-protein cross-links (DPCs).

Transient DPCs between histones and aldehyde containing modified nucleotides have been reported. For instance, reversible Schiff base formation between histones and 5-formyl-2'-deoxycytidine (5fC, Scheme 2) have been detected in NCPs and in isolated cellular DNA.[8] DPCs derived from 5fC adversely affect replication in the test tube and in mammalian cells.[9] Transient DPCs are also formed between abasic sites (AP) and related oxidized abasic

lesions (e.g. C4-AP, DOB) in NCPs (Scheme 1, 2).[5d, 10] The Schiff bases are intermediates on the path to β-elimination products, whose rate constants are accelerated as much as 1500-fold in NCPs compared to in free DNA.[5d]

NCPs containing T-al in 3 representative regions (positions 73, 89 or 158) were prepared (Figure 1A).[11] Position 89 was chosen because it is a well-established hot-spot for DNA damaging agents in a region where the DNA is kinked (Figure 1B).[12] In addition, the lysine-rich histone H4 tail is in close proximity to damaged nucleotides at position 89 and has been shown to play a major role in catalyzing the β-elimination chemistry of abasic sites.[5d, 10] Position 73 is in the region of the NCP dyad axis where the DNA is tightly held and is accessible to the tails of H2A, H3, and H4.[10c] In contrast, none of the histone tails are proximal to position 158.

Oligonucleotides containing a 5'-terminal T-al precursor (**2**) were synthesized as previously described from **1** (Scheme 3).[7] The overall nucleosomal DNA sequence was based on the Widom 601 strong positioning nucleosome.[13] The requisite denaturing PAGE purified oligonucleotides for NCP construction were prepared by 5'-phosphorylating chemically synthesized oligonucleotides, followed by splint mediated ligation using T4 DNA ligase.[14] For reasons described below, the T-al containing oligonucleotide was prepared containing an internal ^{32}P-label. *Xenopus laevis* histone proteins, including H4 variants, were expressed in *E. coli* and purified by ion exchange chromatography.[10c, 15] The NCPs were reconstituted via the salt-dilution method, and characterized by native gel electrophoresis and DNAse I footprinting.[15] T-al was generated immediately prior to experiments in free DNA or NCPs via $NaIO_4$ (5 mM) treatment. Excess periodate was reduced with sodium sulfite (25 mM). The extent of conversion to T-al was determined by measuring the extent of cleavage in a DNA aliquot treated with NaOH (0.1 M, 37 °C, 1 h). To achieve sufficient resolution of T-al from its elimination product, aliquots were treated with a restriction enzyme prior to denaturing PAGE analysis.

The rate constant for T-al elimination (37 °C, pH 7.5) was measured in NCPs and in free DNA for comparison (Table 1). The half-lives at positions 89 (T-al^{89}) and 158 (T-al^{158}) were both between 6 and 7 days. However, T-al at position 73 (T-al^{73}) underwent elimination in the free ternary complex approximately one-half as rapidly ($t_{1/2}$ > 10 days). T-al is considerably less stable than an abasic site, which undergoes β-elimination in free DNA with a half-life on the order of several weeks (~1000 h).[10a, 10d] T- al^{89} and T-al^{73} react more rapidly in NCPs than in the respective free ternary complexes (Table 1). T-al^{89}, which is proximal to the lysine rich histone H4 tail experiences greater accelerated reactivity (2.6-fold) within the NCP than does T-al^{73}. In contrast, the rate constants for β-elimination from T-al^{158} in the NCP and free DNA are within experimental error of one another. These data suggest that interactions of the alkali-labile T-al lesion with the flexible histone tails are responsible for increases in their rate constants for elimination.

Evidence in support of the active role of the histone proteins in promoting T-al^{89} elimination in NCPs was initially gleaned from SDS PAGE analysis. A low but approximately constant yield (~3–6%) of DPCs was detected. The equilibrium DPC yield with T-al^{89} was lower than that reported for 5fC.[8a] Incubation of the NCP containing T-al^{89} in the presence of sodium

cyanoborohydride ($NaBH_3CN$) demonstrated that Schiff base formation was rapid (k = 5.4 ± 1.0 × 10^{-4} s^{-1}, $t_{1/2}$ ~23 min) compared to elimination (Figure 2). In contrast, DPCs were not detected when the T-al^{158} containing NCP was incubated in the presence of $NaBH_3CN$. The large dependence of DPC formation on T-al position within the NCP is consistent with that observed for other electrophilic DNA modifications (e.g. AP, 5fC) and the expected importance of lesion proximity to histone tails (Figure 1).[8a, 10c]

Previous studies on NCPs containing abasic sites or 5fC at position 89 indicated that histone H4 reacts with these modifications.[10a-c, 16] Indeed, histone H4 was the only protein detected by Western blot analysis following isolation of stabilized ($NaBH_3CN$) DPCs formed in NCPs containing T-al^{89} (Figure S5). Subsequent experiments utilizing histone H4 variants within NCPs containing T-al^{89} revealed that the N-terminal tail was largely responsible for the accelerated rate constant for β-elimination (k_{Dec}, Table 2). Mutating histidine 18 to alanine (H18A) or the 5 lysines in the histone H4 tail to arginine (K5,8,12,16,20R) resulted in similar decreases in k_{Dec}. T-al^{89} reactivity within each of these NCPs was intermediate that within the NCP containing wild type histone H4 and free DNA. When these mutations were combined (K5,8,12,16,20R + H18A) the reactivity of T-al^{89} within the NCP was even less than in free DNA. Although it is unclear why this is so, one possibility is that the guanidinium ions in arginine residues coordinate to the T-al carbonyl. There is ample precedent for organocatalysis of carbonyl chemistry by guanidinium groups.[17] Stabilization of the ground state could lead to a deceleration in the observed rate constant, particularly in conjunction with the replacement of histidine 18, a potential base, by alanine (K5,8,12,16,20R + H18A).[18] Regardless of the source, the data clearly indicate that the flexible histone H4 N-terminal tail is predominantly responsible for the accelerated T-al^{89} reactivity in NCPs.

The kinetic data (Table 2) and DPC characterization suggest that the histone protein(s) contribute to T-al elimination in two ways. There is ample evidence that lysine residues increase abasic site reactivity in NCPs via Schiff base formation.[5d, 10] In addition, lysines, as well as histidines, contribute to increased β-elimination via deprotonation.[10d, 19] Although the pK_a of the ε-amines of the lysines are above the pH of the reaction mixture, there is evidence that these residues contribute to β-elimination of abasic sites in NCPs.[10d] However, the acceleration of T-al reactivity in NCPs is considerably smaller than that observed for abasic sites.

In an attempt to shed light on why T-al elimination is more modestly enhanced in NCPs than that of abasic sites, we carried out a series of computational experiments. Although β-elimination can occur through the aldehyde or iminium intermediate, experiments carried out in the presence of $NaBH_3CN$ support the latter. Methyl amine was used as a mimic of the ε-amino group in lysine to form the T-al iminium ion (DPC mimic) and to deprotonate the C4'-proton. DFT computations (B3LYP-D3(BJ)/6–31G*/SMD(H_2O)) predicted that elimination through the iminium intermediate is less energy demanding. Two transition state structures were identified along the reaction coordinate. Proton abstraction ($TS1_{T\text{-}al}$, $\Delta G^{\ddagger}$ = 12.7 kcal/mol) is followed by rate determining phosphate elimination ($TS2_{T\text{-}al}$, $\Delta G^{\ddagger}$ = 21.1 kcal/mol) (Figure 3A). Investigation of elimination from AP revealed a similar reaction profile but the barrier for phosphate elimination was 2.6 kcal/mol lower in energy (18.5 kcal/

mol) than the respective transition state for T-al. A more thorough examination of $TS2_{T\text{-}al}$ and $TS2_{AP}$ revealed that the latter has a short contact (1.87 Å) between hydrogen of the C4-hydroxyl and an oxygen of phosphate group (Figure 3B). Natural bond orbital (NBO) analysis shows two hydrogen bonds between each of the two lone pairs of oxygen and O-H bond (Figure S6) that provide an estimated stabilization energy of ~13.9 kcal/mol, which is clearly missing in $TS2_{T\text{-}al}$ and may contribute significantly to the 2.6 kcal/mol more favourable barrier to β-elimination.

The data presented here indicate that T-al (and perhaps C5'-oxidized aldehydes in general) reactivity in nucleosomal DNA is only modestly increased relative to free DNA in solution. In view of recent observations concerning 5fC and *in vitro* studies indicating that T-al is repaired more slowly than other DNA lesions, C5'-aldehydes may have significant lifetimes in cells and consequential effects on biochemical processes that warrant investigation.[6, 8–9]

Experimental Section

Preparation of ligated oligonucleotides containing T-al precursor.

Chemically synthesized oligonucleotides (100 pmol) were 5'−^{32}P-labeled in a reaction mixture (10 μL) containing 1 × T4 polynucleotide kinase buffer (70 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT), γ−^{32}P-ATP (20 μCi), and T4 polynucleotide kinase (30 U). The reaction mixture was incubated at 37 °C for 4 h and then the reaction was stopped by heating at 65 °C for 30 min. The labelled oligonucleotide was separated from unincorporated γ−^{32}P-ATP via Sephadex-G50 spin column, followed by mixing the ODN containing T-al precursor (200 pmol) and one equivalent of the corresponding splint strand with 1 × T4 DNA ligase buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP). The solution was heated at 95 °C for 2 min, followed by slowly cooling to room temperature. Finally, T4 DNA ligase (800 U, 2 μL) and ATP (1 mM) were added to the solution by maintaining overall 1 × T4 DNA ligase buffer condition. The reaction mixture was incubated at 16 °C for overnight. The ligated products were extracted with phenol:chloroform mixture (equal volume) and purified by denatured PAGE (acrylamide/bisacrylamide = 19:1, 45% urea). The desired bands were excised from the gel and eluted (0.2 M NaCl, 1 mM EDTA) at room temperature overnight. The resulting slurry was filtered using a 10 mL Polyprep Column (BioRad) and concentrated using a 10 kDa Amicon column.

Preparation of NCPs containing T-al precursor.

The internally radiolabeled strand with T-al precursor (typically 20–30 pmol) was mixed with the corresponding flanking and complementary strands (2 equiv.) in the presence of 1 × PBS buffer and 100 mM NaCl. The resulting mixture was heated at 95 °C for 5 min and then slowly cooled to room temperature. A portion of the hybridized solution was used for reconstitution with histone octamer to yield NCP. Salmon sperm DNA (10 μg) and the ternary complex were combined in a siliconized tube (final volume: 10 μL) containing 0.1 mg/mL BSA and 2 M NaCl. Histone octamer (84 pmol) was added and the solution was incubated at room temperature for 30 min. A series of dilutions using nucleosome buffer (10 mM HEPES pH 7.5, 1 mM EDTA, and 0.1 mg/mL BSA) was carried out. Dilution #

(volume in μL, incubation time in min): 1: 10, 60; 2: 6, 60; 3: 6, 60; 4: 10, 30; 5: 10, 30; 6: 20, 30; 7: 50, 30; 8: 100, 30. Any precipitate was pelleted via a brief spin (3 min × 15,000 g, 4 ℃) and the supernatant was transferred to another siliconized tube. All reconstituted nucleosome core particles were stored at 4 °C until further use. The efficiency of reconstitution was determined by nucleoprotein gel electrophoresis (6%, acrylamide/bisacrylamide, 59:1, 0.6 × TBE buffer, run at 4 °C using 0.2 × TBE buffer).

Determining the rate constants describing T-al elimination (k_{Dec}).

Oxidation of the T-al precursor within the NCP was performed using $NaIO_4$ (5 mM) at 37 °C for 1 h. Unreacted $NaIO_4$ was quenched with Na_2SO_3 (25 mM). The reaction mixture was incubated at 37 °C and an aliquot was withdrawn at the appropriate time and immediately cooled at −80 °C. After completing the time course experiment, the samples were treated with $NaBH_4$ (0.1 M) at room temperature for 4 h. Each sample was divided into two portions: one to monitor T-al cleavage and the other one for monitoring DPC formation. For DPC formation, the samples were mixed with 4 × SDS loading buffer (400 mM Tris•HCl, 400 mM DTT, 8% SDS and 40% glycerol) before being subjected to SDS PAGE analysis (10% resolving layer, acrylamide/bisacrylamide = 29:1, 5% stacking layer). For monitoring T-al cleavage, samples were ethanol precipitated twice. The pellet was resuspended in 1 × Cutsmart buffer (50 mM KOAc, 20 mM Tris-acetate, 10 mM $Mg(OAc)_2$, 100 μg/mL BSA, pH 7.9) and treated with restriction enzyme (5 U: Mse1 for position 73, AccI for position 89, and M1uCI/MfeI for position 158), and incubated at 37 °C overnight. The samples were analyzed by 20% denaturing PAGE (acrylamide/bisacrylamide = 19:1, 7 M urea). The extent of T-al reaction was corrected for any unoxidized precursor, which was quantified by treating an aliquot with NaOH (0.1 M, 1 h, 37 °C) and measuring the amount of uncleaved DNA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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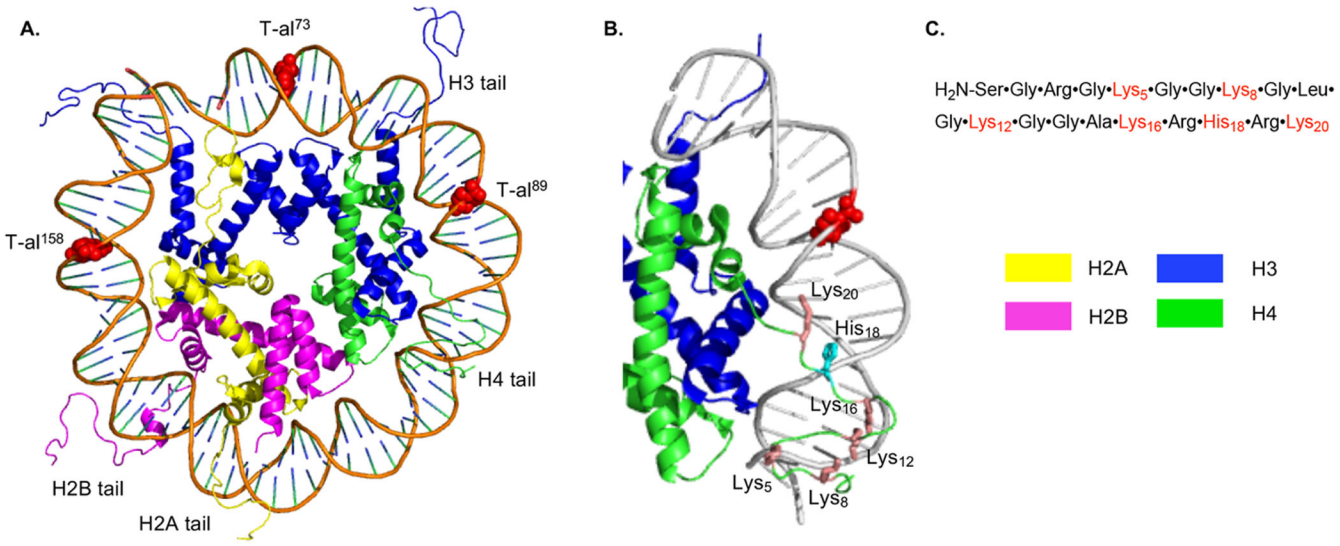


Figure 1.

Nucleosome core particle structure. (A.) Overall structure showing T-al positioning. (A single gyre is shown for clarity.) (B.) Histone H4 residues proximal to T-al^{89}. Lysine side chains, salmon; Histidine side chains, cyan. (C.) Histone H4 N-terminal tail sequence. Positions at which T-al is produced are shown as red spheres. Structural data are from PDB: 1kx5.

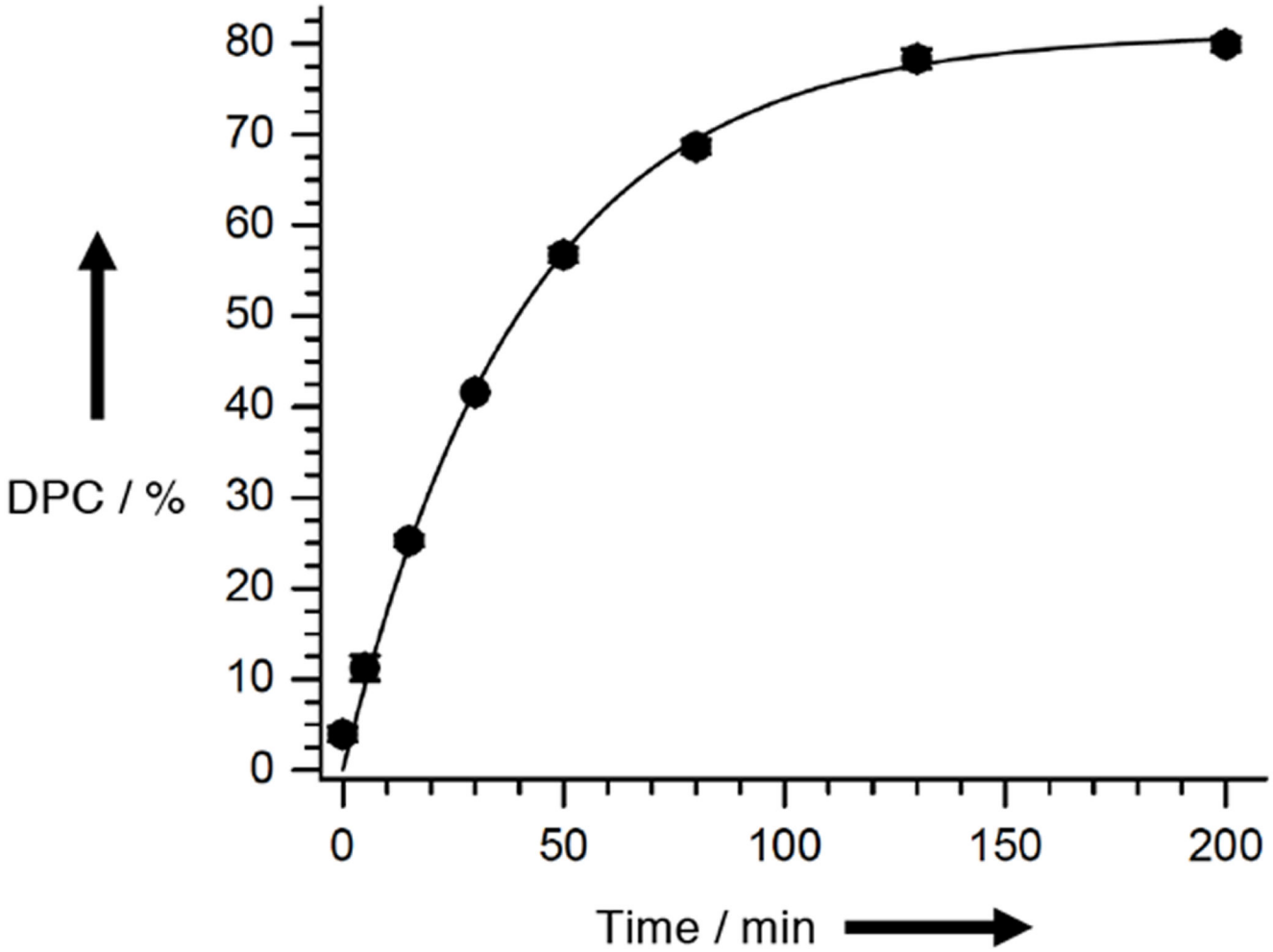


Figure 2.

DNA-protein cross-link (DPC) growth as a function of time when NCP containing T-al[89] is incubated in the presence of $NaBH_3CN$ (10 mM).

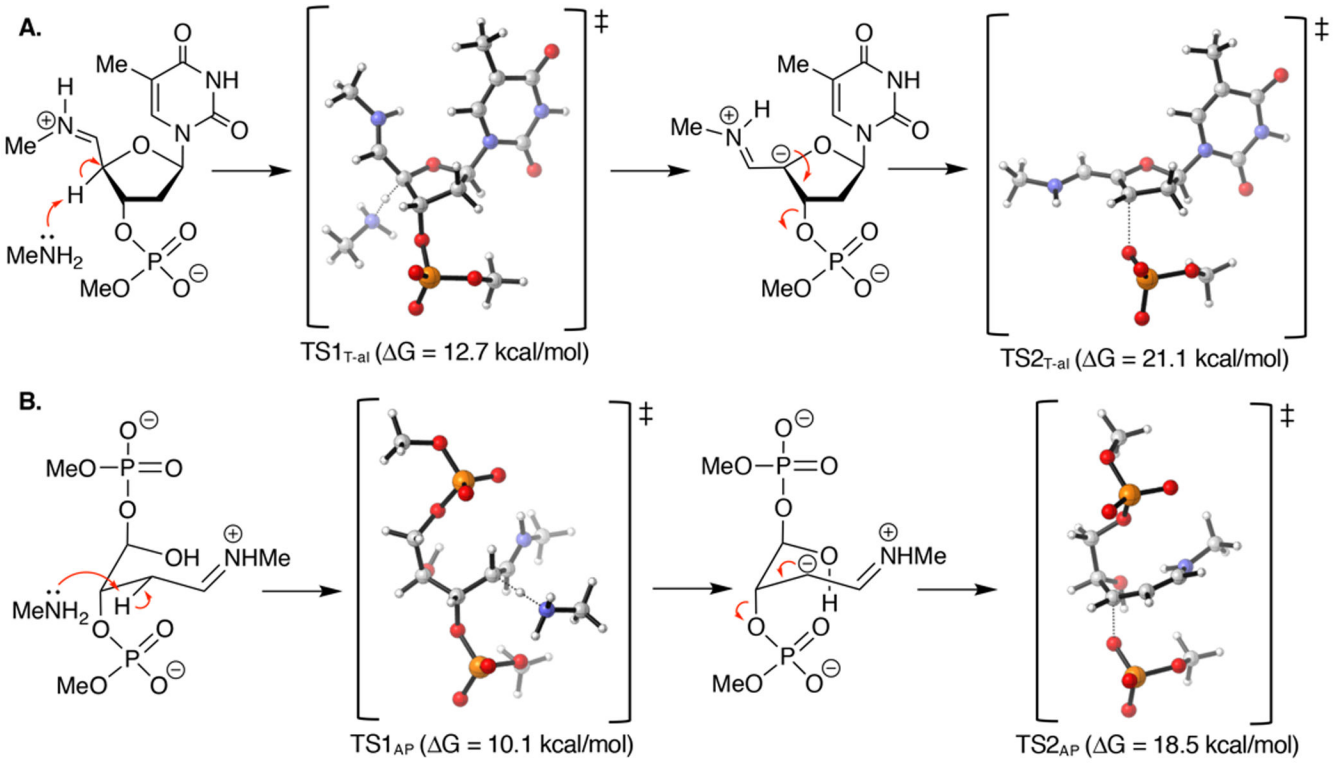
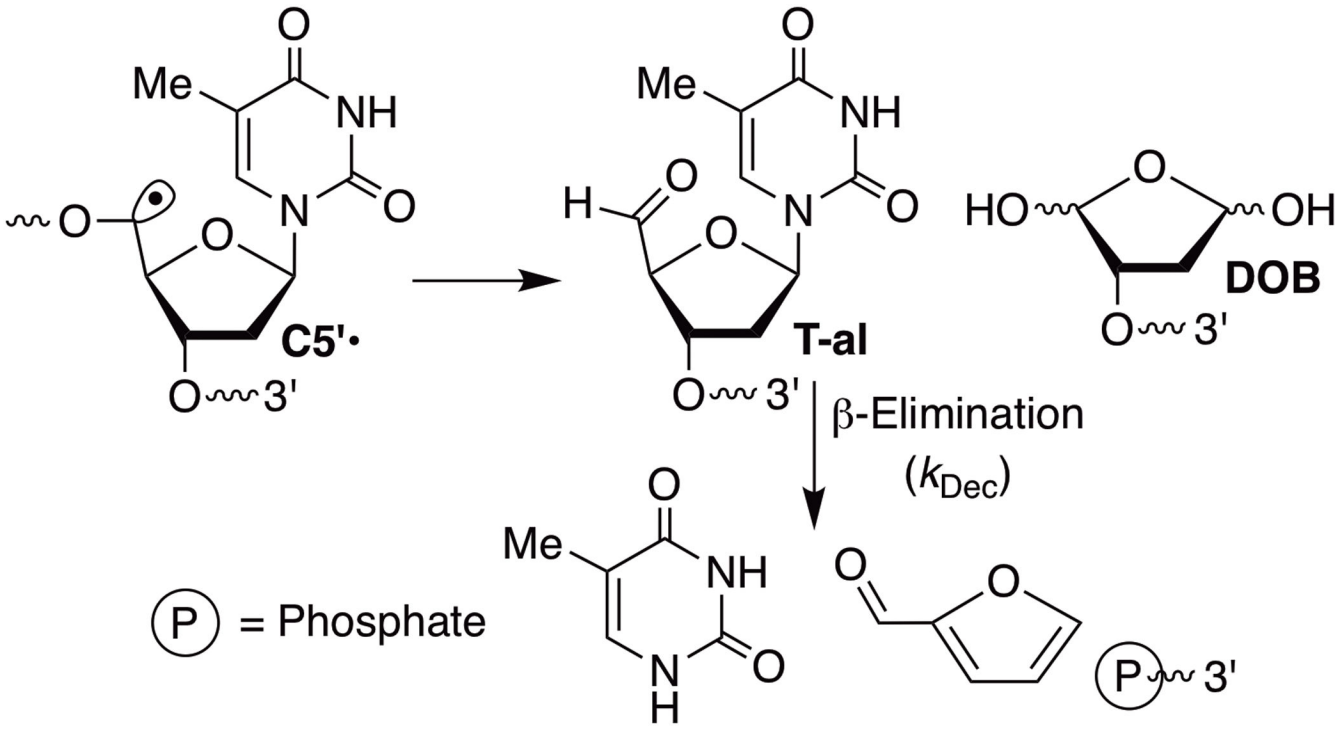
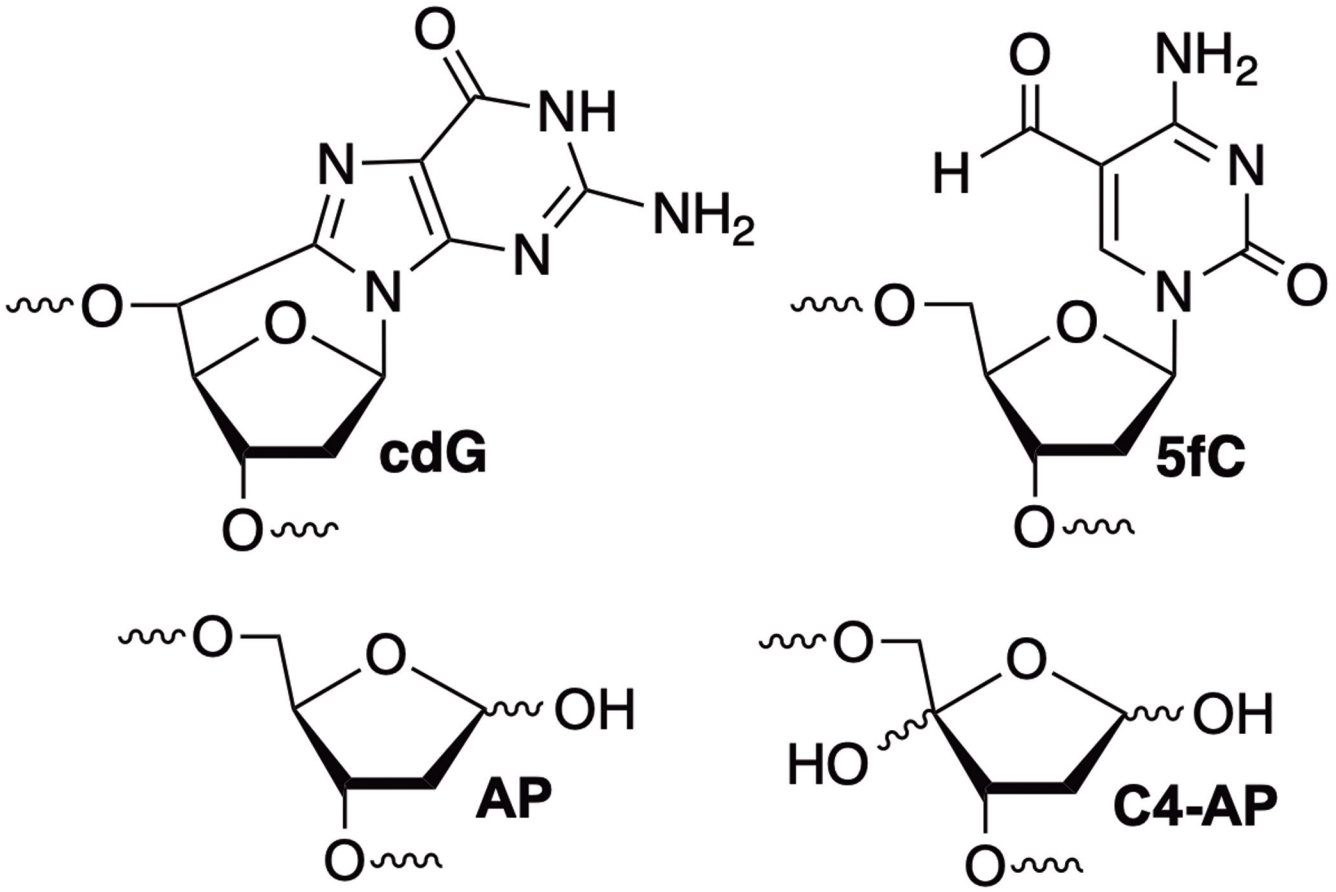


Figure 3.

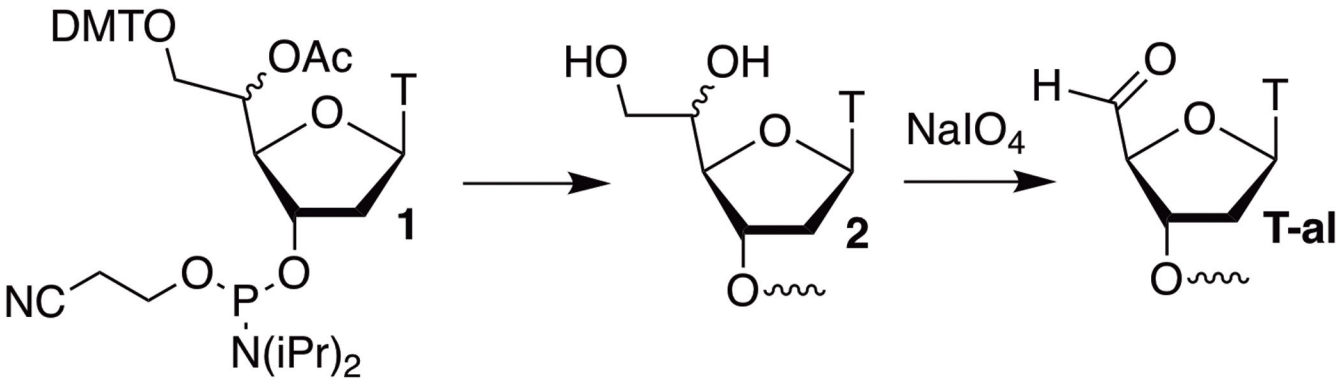
Mechanism of β-elimination from T-al or AP in DNA. A) Reaction sequence for T-al cleavage. B) Reaction sequence for AP cleavage.



Scheme 1.
C5’-Oxidation of thymidine and strand scission in DNA.



Scheme 2.
DNA modifications.



Scheme 3.
Independent generation of T-al in DNA.

Table 1.

T-al reactivity in free DNA and nucleosome core particles.

	k_{Dec} [10^{-7} s^{-1}][a]		
NCP Position	**Free DNA[b]**	**NCP[b]**	**Rel. rate[c]**
73	7.7 ± 0.3 (3)	12.4 ± 0.6 (3)	1.6 ± 0.1
89	11.9 ± 0.6 (2)	30.6 ± 3.4 (3)	2.6 ± 0.3
158	13.4 ± 2.0 (3)	10.8 ± 1.1 (3)	0.8 ± 0.2

[a] Values presented are the average ± std. dev. of two or three experiments, each in triplicate.

[b] Number of experiments in parentheses.

[c] Rel. rate = k_{Dec} (NCP) / k_{Dec} (Free DNA).

Table 2.

T-al[89] reactivity in nucleosome core particles comprised of histone H4 variants.

Histone H4 variant	k_{Dec} [10^{-7} s^{-1}][a]	$t_{1/2}$ (days)
H18A	16.4 ± 1.1	4.9
K5,8,12,16,20R	19.5 ± 1.0	4.1
K5,8,12,16,20R + H18A	8.1 ± 0.4	10.0

[a]Values presented are the average ± std. dev. of three experiments, each in triplicate.