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Oxidative Damage of Thymidines by the Atmospheric Free-Radical Oxidant $NO_3^{\bullet}$

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Analysis of the products formed in the reaction of nitrate radicals, $NO_3^{\bullet}$, with the *N*- and *O*-methylated and acetylated thymidines **1a** and **1b** revealed, for the first time, insight regarding how this important atmospheric free-radical oxidant can cause irreversible damage to DNA building blocks. Mechanistic studies indicated that the initial reaction step likely proceeds via $NO_3^{\bullet}$ induced electron transfer at the pyrimidine ring, followed by deprotonation of the methyl group at C5. The oxidation ultimately leads to formation of nitrates **2**, aldehydes **4** and, in the case of high [$NO_3^{\bullet}$], also to carboxylic acids **5**. In addition to this, through a very minor pathway, loss of the methyl group at C5 also occurred to give the respective 2′-deoxyuridines **6**. The nitrates **2** are highly labile compounds that undergo rapid hydrolysis during work-up and purification of the reaction mixtures, which could lead to serious misinterpretation of the experimental findings and reaction mechanism. Products resulting from $NO_3^{\bullet}$ addition to the C5=C6 double bond in the pyrimidine ring were not observed. Also, no reaction of $NO_3^{\bullet}$ with the 2′-deoxyribose moiety was detected.

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Introduction

Aging processes and several disease states such as cancer can result from DNA damage, which could be induced by exposure of DNA to a variety of oxidizing agents, leading to nucleotide damage and strand breaks. The electron-rich purine and pyrimidine heterocycles are prime targets for these oxidations, which often involve free radical species. Although the initial radical attack usually does not lead to direct DNA strand scission, it may lay the foundation for a site-specific cleavage in a subsequent chemical step. The role of several *O*-centred oxidizing radicals and radical ions, such as hydroxyl, alkoxyl, peroxyl, superoxide, and sulfate radicals (e.g. $HO^{\bullet}$, $RO^{\bullet}$, $ROO^{\bullet}$, $O_2^{\bullet-}$, and $SO_4^{\bullet-}$, respectively) in nucleobase damage has been extensively studied.[1–13] It has also been shown that oxidation products of the thymine base constitute the majority of DNA damage resulting from ionizing radiation. For example, the reaction of $HO^{\bullet}$ with nucleosides usually occurs via initial radical addition to double bonds, which in the case of thymidines leads to products characterized by a saturated C5–C6 bond.[14–18] In contrast to this, the less reactive $ROO^{\bullet}$ are suggested to react with thymidine by hydrogen abstraction at the C5 methyl group, which ultimately leads to formation of 5-formyl-2′-deoxyuridine.[12]

The nitrate radical ($NO_3^{\bullet}$) is the most important night-time oxidant, which is formed in the atmosphere by reaction involving the environmental pollutants nitrogen dioxide, $NO_2^{\bullet}$, and ozone, O_3 (Eqn 1):

$$NO_2^{\bullet} + O_3 \rightarrow NO_3^{\bullet} + O_2 \qquad (1)$$

Although numerous investigations have been performed to obtain a fundamental understanding of the role of $NO_3^{\bullet}$ in the

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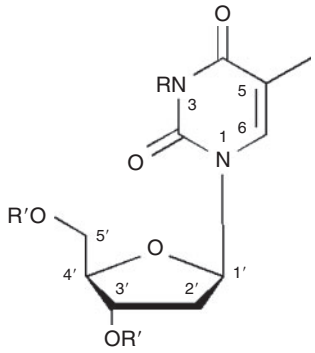
tropospheric transformation processes,[19–22] there is only very limited information available on the damage of biomolecules caused by reaction with $NO_3^\bullet$. In contrast to other free radical species, which generally react via one pathway only, $NO_3^\bullet$ exhibits a multifaceted reaction pattern, e.g. (i) hydrogen atom abstraction (HAT), (ii) addition to π systems, and (iii) electron transfer (ET). Thus, every class of organic compounds is, in principle, susceptible to attack by $NO_3^\bullet$. The oxidation power of $NO_3^\bullet$, which is higher than that of any biochemical radical and non-radical oxidant, has potentially serious implications for biological systems. For example, we have recently shown that aromatic amino acids are rapidly and irreversibly damaged by $NO_3^\bullet$ induced oxidation, and from the products formed in these reactions it might be suggested that atmospheric $NO_3^\bullet$ could be the culprit in certain pollution-derived diseases of the respiratory tract.[23,24] We have also demonstrated that $NO_3^\bullet$ can initiate oxidative repair of pyrimidine cyclobutane dimers, which are the most important reaction products in DNA caused by UV irradiation.[25,26] Although it is not clear at the moment, whether $NO_3^\bullet$ can actually enter biological systems beyond biosurfaces exposed to the atmospheric environment, studies of $NO_3^\bullet$ induced oxidative damage in DNA can reveal important insight into the mechanism of these processes. Knowledge of the latter is of fundamental importance since, in reactions of nucleosides or DNA with various different oxidizing species, certain intermediates appear to be common to many systems, and as such the determination of one mechanistic pathway will help to elucidate others.

In this paper, we wish to report the results of the first product and mechanistic study of the reaction of $NO_3^\bullet$ with the *N*- and *O*-methylated and acetylated thymidines **1a** and **1b**, respectively (Fig. 1). These two pyrimidines were chosen to explore the role of electron density at the heterocyclic base on the degree of oxidative damage. Thus, whereas in thymidine **1a** *N*-methylation leads to an increased electron density at the base, the *O*-acetylated thymidine **1b** features an unsubstituted imide N–H bond as it occurs in DNA. The hydroxyl groups in the 2′-deoxyribose moiety were protected by methylation or acetylation to mimic the diester linkage in DNA.

$NO_3^\bullet$ was generated in situ, in the presence of the respective thymidines **1a**/**b**, by photolyzing a solution of ammonium cerium(IV)nitrate (CAN) in acetonitrile, which gives $NO_3^\bullet$ through a photoinduced electron transfer reaction (Eqn 2).[19,23,27,28]

$$(NH_4)_2Ce(NO_3)_6 + h\nu \rightarrow (NH_4)_2Ce(NO_3)_5 + NO_3^\bullet \qquad (2)$$



1a R = R′ = Me
1b R = H, R′ = Ac

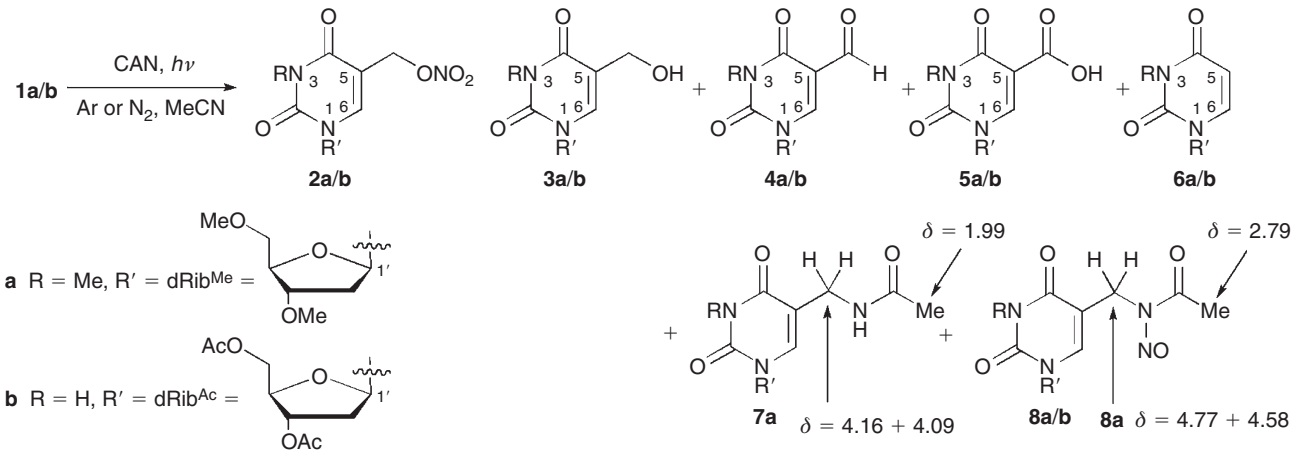
Fig. 1. Thymidines studied in this work.

Results and Discussion

Product Studies

Using CAN both in excess or as a minor compound, respectively, the irradiations were usually performed until the characteristic yellow-orange colour of CAN had disappeared, indicating complete consumption of the radical precursor (typically 2–3.5 h in the reactions with excess CAN). Identification of the products was performed by isolation and purification through repeated column chromatography or by HPLC, followed by spectroscopic characterization. Further details are given in the Experimental section.

Scheme 1 details the products obtained in the reaction of CAN with **1a** and **1b**, respectively, under different experimental conditions. The large variety of products is a clear indication that the reactions are complex. Most importantly, we found that the outcome was very sensitive to the method of reaction analysis and purification of the products, which could lead to serious misinterpretation of the reaction mechanism. These findings will be discussed in detail in the section titled 'Mechanistic Considerations' of this paper.



Scheme 1.

Table 1 compiles the products of the individual reactions, which were performed with an excess of CAN ([CAN]/[**1a** or **1b**]; 2/1). Under these conditions, consumption of the respective thymidines was not complete, and the yields are therefore based on consumed starting material.

The yields were obtained from quantitative ^{1}H NMR analysis of the reaction mixture after filtration through a short silica gel column to remove insoluble inorganic material, using a measured amount of benzene as internal standard. The ^{1}H NMR chemical shifts of benzene (δ 7.36 ppm) and those of the starting thymidines and the various products, showed some characteristic differences, specifically the protons at the C5 side chain, the vinyl proton H6, and the anomeric proton H1′, which are listed in Table 2. This enabled us to use these chemical shifts as a marker to determine the absolute composition of the reaction mixture from signal integration relative to that of the standard.

All reaction products were derived from oxidation of the methyl group at C5, leading to formation of the respective nitrates (**2a**/**b**), alcohols (**3a**/**b**), and aldehydes (**4a**/**b**) in varying yields. A value of ≤3% indicates that yields were below the detection limit of the ^{1}H NMR spectrometer. No products arising from a reaction of $NO_3^\bullet$ with the 2′-deoxyribose moiety were found, which could only proceed via $NO_3^\bullet$ induced HAT from a methylene group adjacent to an oxygen atom. Also, no products resulting from radical addition to the C5=C6 double bond in the pyrimidine were formed.

In the reaction of the permethylated thymidine **1a** with $NO_3^\bullet$, formation of aldehyde **4a** with a yield of 44% was the major pathway. Conversely, the nitrate **2a** was obtained in 23% yield, together with a minor amount of alcohol **3a**, which we believe results from hydrolysis of nitrate **2a** during work-up (see below). Indeed, **2a** turned out to be highly labile and could not be obtained as a pure compound. Therefore, identification and spectroscopic characterization could only be performed from a mixture containing also aldehyde **4a** and by comparing the data with those of the slightly more stable nitrate **2b**, which was formed in the reaction of thymidine **1b** with $NO_3^\bullet$ and could be isolated in pure form. As can be seen from Table 2, the most characteristic and indicative signals in the ^{1}H NMR spectrum of nitrates **2a**/**b** are the methylene protons of the C5 substituent, which exhibit a strong downfield shift to δ 5.29 ppm (**2a** and **2b**) and 5.21 ppm (**2a**) or 5.22 ppm (**2b**) ppm, respectively, resulting from the electron-withdrawing nitrate substituent. For the reaction involving thymidine **1a**, it was further exemplarily shown that with very high local $NO_3^\bullet$ concentrations, which could be produced by using a focussed high-pressure mercury lamp, oxidation to the carboxylic acid **5a** occurred.

The *O*-acetylated thymidine **1b** which possessed a free imide N–H bond, so that the pyrimidine ring system is less electron rich compared with **1a**, reacted with $NO_3^\bullet$ to form predominantly nitrate **2b** (59%), whereas the corresponding aldehyde **4b** was the minor product (19%). Alcohol **3b** was not found in the reaction mixture. In order to obtain spectroscopic data for **3b**, the latter was independently prepared from nitrate **2b** through acid-mediated hydrolysis (refer to Experimental section herein).

Interestingly, only after very careful purification of the reaction mixtures by preparative HPLC, could very small amounts of the 2′-deoxyuridines **6a** and **6b** be isolated, which likely result from oxidative demethylation at C5. The yield of these compounds was too low to be detected in the ^{1}H NMR

Table 1. Products and yields of the reaction of $NO_3^\bullet$ with pyrimidines 1a and 1b under anaerobic conditions with [CAN]/[1a or 1b] ~2[A]

Entry	Pyrimidine	Products and yields[A]				
1	**1a**	**2a**: 23%	**3a**: 6%[B]	**4a**: 44%	**5a**: ≤3%[C]	**6a**: ≤3%[C]
2	**1b**	**2b**: 59%	**3b**: ≤3%[C]	**4b**: 19%	**5b**: ≤3%[C]	**6b**: ≤3%[C]

[A]Determined by quantitative ^{1}H NMR analysis of the reaction mixture (see text).
[B]Isolated yield.
[C]Yield below detection limit of ^{1}H NMR spectrometer.

Table 2. Characteristic ^{1}H NMR chemical shifts (δ) in ppm of thymidines 1 and products 2–6 formed in the reaction with $NO_3^\bullet$
n.d., not determined

Signal	Compound					
	1	**2**	**3**	**4**	**5**	**6**
(a) Reaction of $NO_3^\bullet$ with **1a**						
5-$CH_{(3-n)}$X	1.92[A]	5.29, 5.21[B]	4.47, 4.35[C]	9.97[D]	–[E]	5.77[F]
H6	7.59	8.20	7.88	8.81	9.21	7.72
H1′	6.31	6.30	6.32	6.22	6.29	6.28
(b) Reaction of $NO_3^\bullet$ with **1b**						
5-$CH_{(3-n)}$X	1.93[A]	5.29, 5.22[B]	4.47, 4.39[C]	9.95[D]	n.d.	5.80[F]
H6	7.29	7.81	7.59	8.47	n.d.	7.53
H1′	6.32	6.26	6.31	6.28	n.d.	6.26

[A]$n = 0$.
[B]$n = 1$, X = ONO_2.
[C]$n = 1$, X = OH.
[D]$n = 2$, X = (=O).
[E]$n = 3$, X = (=O)OH.
[F]H5.

spectra of the reaction mixtures. However, discussion of possible pathways for their formation will follow in 'Mechanistic Considerations'.

The low stability of the nitrates **2a**/**b** poses a serious problem with regards to identification of the primary products formed in the reaction of $NO_3^\bullet$ with thymidines **1a**/**b**. We have found that hydrolysis of **2a**/**b** occurs quantitatively during separation of the reaction products by preparative HPLC, if a non-buffered solvent system is used. In those cases, we could only isolate the respective alcohols **3** and the acetamides **7** and **8**. The latter two products result from a Ritter reaction involving the solvent acetonitrile. The mechanism and implications of these secondary reactions will be discussed in Mechanistic Considerations.

In order to obtain insight into the influence of $[NO_3^\bullet]$ on the composition of the product mixture, the reaction of **1a** and **1b** with $NO_3^\bullet$ was also explored with the pyrimidine in at least fourfold excess. Qualitative ^{1}H NMR analysis of the reaction mixtures revealed that under these conditions only formation of the respective nitrates **2a** and **2b** occurred, whereas higher oxidation products, such as aldehydes, were not formed (data not shown).

Mechanistic Considerations

The results of the product analysis have clearly shown that reaction of $NO_3^\bullet$ with the pyrimidines **1a** and **1b** occurs exclusively at the methyl substituent at C5. For the reaction of $NO_3^\bullet$ with thymidine **1a**, which was performed with excess $NO_3^\bullet$ (e.g. [CAN]/[**1a**]; 6/1) under exclusion of oxygen, we have used analytical HPLC to obtain qualitative concentration–time profiles for the various reaction products (Fig. 2). The HPLC runs were carried out using acetonitrile/water as solvent system, which was buffered with 0.05 M triethylammonium acetate. The data were obtained from the relative peak areas of the product signals monitored at λ 260 nm.[29] These experiments clearly revealed that formation of **2a** occurred first, whereas the aldehyde **4a** and carboxylic acid **5a** appeared only after induction periods, indicative of secondary products. However, the lack of an induction period for formation of the alcohol **3a** supports our assumption that **3a** results only from hydrolysis of nitrate **2a** during HPLC analysis and is not directly formed in the reaction of $NO_3^\bullet$ with **1a**.

An analogous experiment performed with [CAN]/[**1a**] (2/1) in the presence of oxygen showed that formation of both aldehyde **4a** and carboxylic acid **5a** occurred much faster at the expense of nitrate **2a** (and alcohol **3a**) production, compared with the experiments in the absence of oxygen, despite the lower concentration of $NO_3^\bullet$ (data not shown). This indicates that some reaction intermediates are susceptible to trapping by oxygen.

Due to the high oxidation power of $NO_3^\bullet$ [E^0 ($NO_3^\bullet/NO_3^-$) = 2.3 V versus NHE],[28,30,31] we believe that the reaction is initiated by ET at the pyrimidine ring, which likely proceeds via an addition–elimination pathway, as has been suggested for the reaction of $NO_3^\bullet$ with alkylaromatic compounds.[32] Scheme 2 outlines the proposed mechanism for formation of the nitrate **2a** using the reaction of $NO_3^\bullet$ with thymidine **1a** as example.

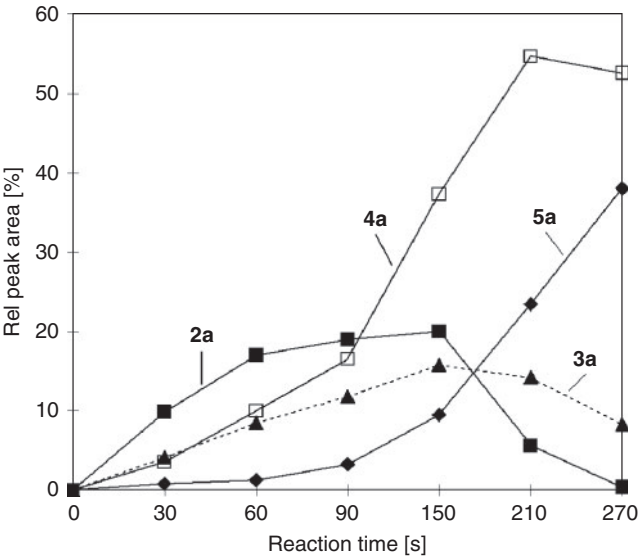
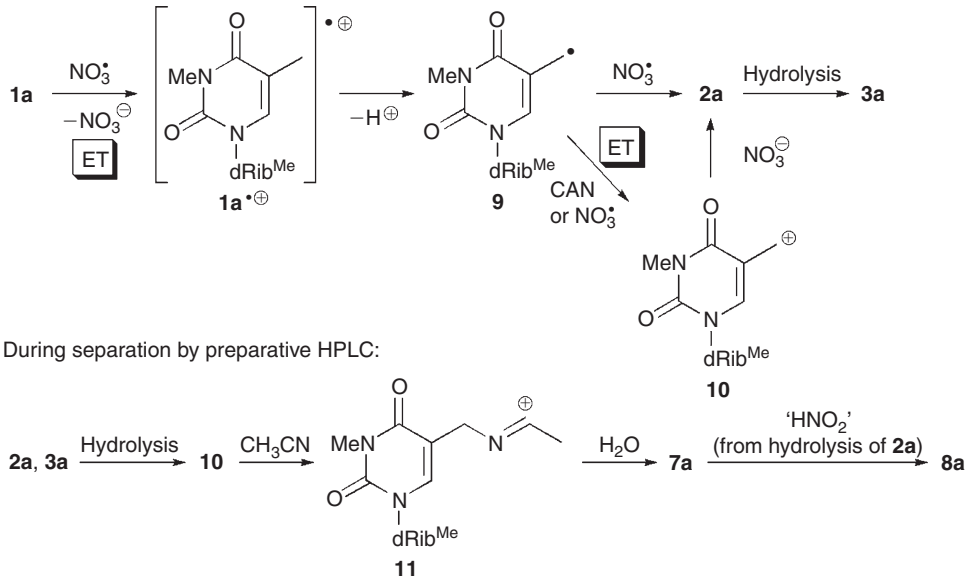


Fig. 2. Qualitative concentration–time profiles for the products formed in the reaction of $NO_3^\bullet$ with thymidine **1a** under anaerobic conditions.



Scheme 2.

According to this, the initially formed radical cation **1a**$^{\bullet+}$ undergoes deprotonation to give benzyl-type radical **9**,[9] in analogy to the mechanism of the $NO_3^\bullet$ induced oxidation of aromatic amino acids. The latter reactions have been shown to proceed through an intermediate aryl radical cation, which can be directly trapped if the reaction is performed in the presence of $NO_2^\bullet$ to give nitroaromatic products.[24] In the absence of $NO_2^\bullet$ stabilization through deprotonation occurs, which leads to products with an oxidized benzylic site.[23] Principally, the reaction of $NO_3^\bullet$ with **1a** could also be initiated through HAT from the C5 methyl group, which would give radical intermediate **9** in a single step. However, HAT reactions by $NO_3^\bullet$ are usually much slower than ET.[19–22] The suggested ET is further supported by the finding that reaction of **1a** with CAN in the absence of light (e.g. absence of $NO_3^\bullet$) leads to the same products, but in a significantly slower reaction, which is in agreement with the much lower redox potential of CAN [E^0 (Ce^{4+}/Ce^{3+}) = 1.61 V versus NHE].[33]

Formation of nitrate **2a** could principally occur via two different pathways, e.g. through direct trapping of **9** by $NO_3^\bullet$, or in a two-step process, first by $NO_3^\bullet$ or CAN induced oxidation, followed by quenching of the resulting benzyl-type cation **10** through ligand transfer from CAN. Subsequent hydrolysis of **2a** leads to alcohol **3a**. Indeed, in light of the observed acetamides **7a** and **8a** (Scheme 1), it could be interpreted that the reaction leading to nitrate **2a** proceeds via an intermediate cation **10**, which is trapped by the solvent acetonitrile through a Ritter reaction.[34,35] However, the acetamides **7**/**8** were *only* obtained, when product isolation was performed by HPLC in an unbuffered solvent system, in which case the nitrates were *not* observed as products. On the other hand, HPLC analysis of the reaction mixture under buffered conditions did not reveal any acetamides. We conclude therefore, that the Ritter reaction involving cation **10** occurs only during purification of the reaction mixture through hydrolysis of nitrate **2a** (and to some extent possibly also the alcohol **3a**), and that formation of a cationic intermediate of type **10** in the actual reaction of $NO_3^\bullet$ with thymidines is, if at all, only a very minor pathway. Formation of the *N*-nitroso acetamide **8a** is believed to proceed also on the HPLC column through *N*-nitrosation of **7a** by nitrous acid, which could be intermediately formed during hydrolysis of nitrate **2a**. The ^{1}H NMR spectrum of **8a** is characterized by significant downfield shifts of the methylene protons at C5 by ~0.5–0.6 ppm and of the acetyl protons by 0.8 ppm, compared with the free acetamide **7a** (see Scheme 1), which is in line with the electron-withdrawing nature of the nitroso group.[24]

It is quite apparent from these findings that analysis of the products formed in the reaction of $NO_3^\bullet$ (and potentially also other oxidizing radicals) with thymidines must be carried out with great care so that crucial details, which could lead to a misinterpretation of the reaction mechanism, are not missed. It is only because we have performed our analytical studies under a variety of different conditions that we are able to distinguish between direct reaction products and products arising from post-reaction chemical modification of highly labile compounds.

According to the proposed mechanism in Scheme 2, formation of the nitrate **2a** would require two equivalents of $NO_3^\bullet$. Indeed, our photochemical experiments with CAN as minor compound confirmed this hypothesis by showing, that after complete consumption of CAN, the ratio of nitrate **2a** to unreacted pyrimidine **1a**, [**2a**]/[**1a**], in the ^{1}H NMR spectrum of the reaction mixture was significantly lower than would be expected from the initial reactant ratio [CAN]/[**1a**] for a stoichiometric reaction.

Scheme 3 outlines the assumed mechanism for formation of the aldehyde **4a** from nitrate **2a**, which could proceed without or with involvement of oxygen.

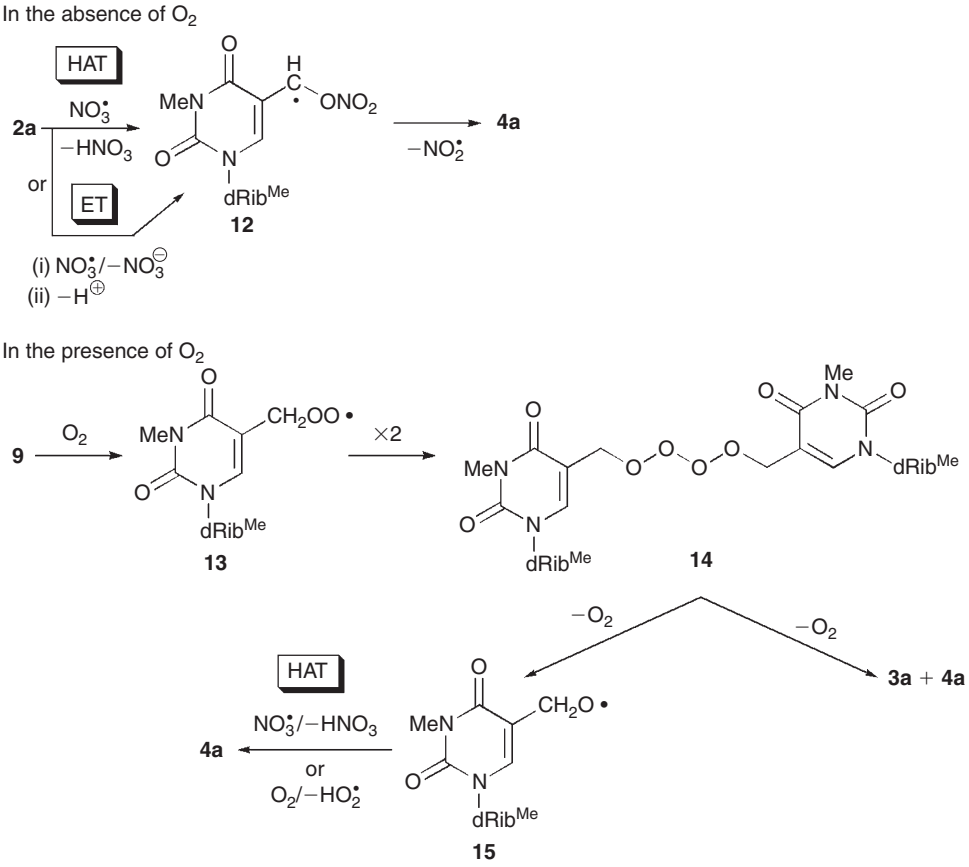
In the absence of oxygen, nitrate **2a** could be converted into the corresponding benzylradical **12** through either a direct HAT by $NO_3^\bullet$,[36] or through a coupled ET/deprotonation pathway, analogous to the initial reaction step. The O–NO_2 bond in **12** is very weak and can undergo rapid β-fragmentation to give aldehyde **4a** with release of $NO_2^\bullet$, which is too unreactive to initiate a radical chain process.[37]

In the presence of oxygen, the radical intermediate **9** could be trapped to give peroxyl radical **13**, which could be transformed into alkoxyl radical **15** through dimerization and release of oxygen. Subsequent conversion of **15** into aldehyde **4a**, could occur through HAT by either $NO_3^\bullet$ or oxygen, respectively. The closed-shell dimer **14** could principally also decompose directly to the alcohol **3a** and aldehyde **4a** through a Russel mechanism involving a cyclic transition state.[38] Thus, the latter pathway could lead to formation of the alcohol **3a** without hydrolysis of the nitrate **2a**. The suggested mechanism involving formation of an intermediate peroxyl radical **13**, which intercepts the pathway leading to nitrate **2a**, is in line with the qualitative finding that less nitrate **2a** and significantly more aldehyde **4a** was obtained when the reaction of $NO_3^\bullet$ with thymidine **1a** was carried out in the presence of oxygen (see above). In addition, formation of peroxyl radical **13** is an additional support for our aforementioned assumption that the reaction of $NO_3^\bullet$ with thymidines does not proceed via a cationic species **10**, as the latter would not be expected to react rapidly with molecular oxygen.

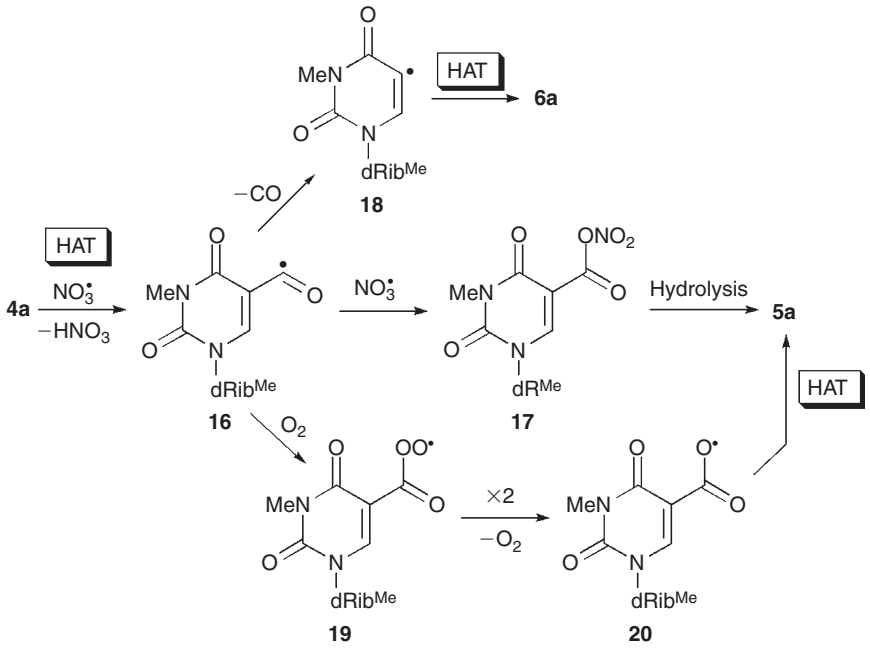
Scheme 4 shows the suggested mechanistic steps that lead to formation of the carboxylic acid **5a**. The mechanism is based on findings from independent experiments, where we demonstrated that aldehyde **4a** can be oxidized to **5a** by $NO_3^\bullet$. This reaction likely proceeds via HAT,[39] where the resulting acyl radical **16** could subsequently be trapped by $NO_3^\bullet$ to give mixed anhydride **17**, which is hydrolyzed to the acid **5a** during the aqueous work-up and/or purification by chromatography. The observed formation of small amounts of the uridine **6a** in the absence of oxygen could be explained through a pathway involving decarbonylation in **16**,[40] followed by reduction of the resulting highly reactive vinyl radical **18** through HAT (likely from the solvent). The finding of a more rapid formation of carboxylic acid **5a** in the presence of oxygen could be rationalized by a mechanism, where acyl radical **16** is trapped to give acylperoxyl radical **19**, which could subsequently be transformed into the acyloxyl radical **20** through dimerization and expulsion of oxygen. HAT from the solvent then leads to the acid **5a**. It cannot be excluded, however, that the radical intermediate **20** could also undergo decarboxylation to give vinyl radical **18**, which would represent an additional, oxygen-dependent pathway to uridine **6a** (not shown).

Conclusions

This work is the first product study of the reaction of the atmospheric free radical oxidant $NO_3^\bullet$ with thymidines. Using the *N*- and *O*-methylated and acetylated thymidines **1a** and **1b** as model systems for thymidine nucleotides, it was found that the reaction occurred exclusively at the methyl side chain at C5, which led to formation of nitrates **2a**/**b** and aldehydes **4a**/**b** as the major products. Using the reaction with thymidine **1a** as



Scheme 3.



Scheme 4.

example, carboxylic acid **5a** was also produced at high concentrations of $NO_3^\bullet$. In addition, also very small amounts of 2′-deoxyuridines **6a**/**b** were formed through demethylation at C5. In contrast to the reaction of thymines and thymidines with other *O*-centred radicals, we have no indications for products arising from $NO_3^\bullet$ addition to the C5=C6 double bond in the pyrimidine. Also, no reaction of $NO_3^\bullet$ with the 2′-deoxyribose moiety in **1a**/**b** was observed.

A thorough discussion of the reaction mechanism revealed that the reaction is very likely initiated by $NO_3^\bullet$ induced ET, which leads to the benzyl-type radical intermediate **9**. The latter has been suggested to be able to transfer damage from the nucleobase to the deoxyribose moiety of an adjacent nucleotide in the same oligonucleotide strand.[41]

The primary products in the reaction of $NO_3^\bullet$ with thymidines, for example nitrates **2**, are highly labile species, which readily undergo hydrolysis to the significantly more stable alcohols **3**. Such a hydrolysis reaction could potentially provide a source for reactive benzyl-type cations **10** under physiological conditions, which is supported by the finding that formation of **10** readily occurs under certain conditions during isolation and purification of the reaction mixtures by HPLC. In our system, the cations **10** were trapped by the solvent acetonitrile to give acetamides **7** and **8** through a Ritter reaction. Since our experimental data clearly indicate that the $NO_3^\bullet$ induced oxidation of thymidines **1** does not involve cation **10**, it is important to note that such a rapid post-reaction modification of products, as was observed in this work, can lead to serious misinterpretation of experimental data and reaction mechanisms, if it remains undiscovered.

In contrast to the nitrates **2a**/**b**, 5-formyl-2′-deoxyuridines of type **4a**/**b** are reasonably stable species, which have been frequently found during radical mediated oxidation of thymidines[13,42,43] and are known to induce point mutations during DNA replication.[44] Our experiments revealed that the rate of $NO_3^\bullet$ induced formation of 5-formyluridine was dependent on the electron density at the pyrimidine ring. In the case of the more electron-rich permethylated thymidine **1a**, the reasonably stable formyluridine **4a** was the major reaction product. Conversely, the reaction of thymidine **1b**, in which the pyrimidine ring has a free imide N–H group and therefore represents the natural conditions of the base in DNA, gave the labile nitrate **2b** as major product under otherwise identical reaction conditions. However, oxidation of thymidine **1** to 5-formyluridine **4** was significantly accelerated in the presence of oxygen.

Clearly, our experimental conditions differed from the typical conditions in biological systems, specifically with regards to reaction solvent. However, it is important to note that the aprotic acetonitrile, which was used as solvent in this work, can reasonably mimic the hydrophobic environment of the nucleobases in DNA.[45] In principle, residual water could influence the reaction outcome in two ways, through (i) trapping of intermediates produced in the reaction of $NO_3^\bullet$ with thymidines, and through (ii) reaction with $NO_3^\bullet$ itself. The first possibility can be excluded since our mechanistic considerations revealed that no cationic intermediates are produced in the reaction of $NO_3^\bullet$ with thymidines, which could be trapped by water acting as a nucleophile. Further, it has been shown that the mechanism of $NO_3^\bullet$ reactions with organic compounds in water is similar to that in organic solvents.[46] Although the reaction of $NO_3^\bullet$ with water is not very fast ($k = 3 \times 10^2$ $M^{-1}\,s^{-1}$ [46]); if it occurs, highly reactive $HO^\bullet$ could be formed, which are known to react rapidly with thymidines and can induce strand cleavage in DNA.[1–4,7,8] Future work will be directed towards addressing details of the mechanism of the oxidative damage of nucleosides by $NO_3^\bullet$, as well as the secondary effects resulting from the presence of residual water, by studying absolute rate constants and reaction transients using nanosecond laser flash photolysis.

Experimental

^{1}H NMR spectra were recorded on a Bruker AM 300, DRX 500, DRX 600, and a Varian Unity Inova 500 spectrometer operating at 300, 500, or 600 MHz, respectively. ^{13}C NMR spectra were obtained on the same instruments operating at 75.5, 125.8, or 150.9 MHz, respectively. Mass spectra were recorded on a Finnigan MAT 8200 instrument at 70 eV ionizing potential. Isobutane was used for chemical ionization (CI). HR-MS was conducted by ionizing the samples via electrospray ionization into a Thermo-Finnigan LTQ FT-ICR hybrid mass spectrometer or an Agilent 6520 LC/Q-TOF mass spectrometer with an electrospray ionizing source coupled to an Agilent 1100 LC system.

HPLC columns and conditions: (a) analytical: Merck LiChrospher 100e, RP18, 5 µm, 250 × 4 mm, 1 mL min^{-1}; (b) semi-preparative: Machery-Nagel LiChrospher 100e, RP18, 5 µm, 200 × 10 mm, 4 mL min^{-1}; solvents: acetonitrile (A); water/acetonitrile (1%) (B); gradient: 0% A → 30% A in 40 min; (C) preparative: Phenomenex, C18, 5 µm, 150 × 21.2 mm, 8 mL min^{-1}; solvents: acetonitrile (A); water (B); gradient: 0% A → 100% A in 2–3 h.

The irradiations were performed under a continuous gas flow (nitrogen, argon, or oxygen) in either a Rayonet Photochemical Reactor (λ 300 or 350 nm) or with a mercury lamp, where UV light below λ 280 nm was eliminated either by a cut-off filter or by conducting the reaction in duran glassware. Before the irradiations, residual oxygen was removed from the reaction mixture by bubbling argon or nitrogen through the solution. Synthesis of **1a** and **1b** was performed according to standard procedures.[47,48]

3′,5′-Di-O-*methyl*-N-*methyl-2′-desoxythymidine (**1a**)*: δ_H ($CDCl_3$, 500 MHz) 7.59 (s, 1H), 6.31 (dd, *J* 7.5, 6.1 Hz, 1H), 4.11 (dd, *J* 5.6, 2.8 Hz, 1H), 3.98 (dt, *J* 5.9, 2.7 Hz, 1H), 3.65 (dd, *J* 10.5, 2.8 Hz, 1H), 3.56 (dd, *J* 10.5, 2.8 Hz, 1H), 3.42 (s, 3H), 3.33 (s, 3H), 3.31 (s, 3H), 2.40 (ddd, *J* 13.7, 6.0, 2.7 Hz, 1H), 2.03 (m, 1H), 1.92 (s, 3H). δ_C ($CDCl_3$, 125 MHz) 163.8, 151.2, 133.9, 109.8, 86.0, 83.8, 81.3, 73.1, 59.2, 57.0, 37.6, 27.9, 13.6. *m/z* (HR-ESI). Calc. for $C_{13}H_{20}N_2O_5$+H: 285.1451. Found: 285.1656. Calc. for $C_{12}{}^{13}CH_{20}N_2O_5$+H: 286.1484. Found: 286.1497.

3′,5′-Di-O-*acetyl-2′-desoxythymidine (**1b**)*: δ_H ($CDCl_3$, 500 MHz) 9.67 (s, 1H), 7.29 (s, 1H), 6.32 (dd, *J* 8.6, 5.7 Hz, 1H), 5.21 (dt, *J* 6.6, 2.2 Hz, 1H), 4.37 (dd, *J* 12.1, 4.2 Hz, 1H), 4.33 (dd, *J* 12.1, 3.3 Hz, 1H), 4.24 (dd, *J* 6.4, 3.4 Hz, 1H), 2.47 (ddd, *J* 14.2, 5.7, 1.9 Hz, 1H), 2.16 (m, 1H), 2.12 (s, 3H), 2.11 (s, 3H), 1.93 (s, 3H). δ_C ($CDCl_3$, 125 MHz) 170.6, 170.3, 164.1, 150.5, 134.8, 111.7, 85.0, 82.3, 74.2, 64.0, 37.7, 21.0, 20.9, 12.8. *m/z* (HR-ESI). Calc. for $C_{14}H_{18}N_2O_7$+H: 327.1192. Found: 327.1190. Calc. for $C_{13}{}^{13}CH_{18}N_2O_7$+H: 328.1226. Found: 328.1222.

Reaction of $NO_3^\bullet$ with Thymidines. Typical Experimental Procedure

Thymidine **1** (0.5 mmol) and CAN (1.0 mmol) were dissolved in 45 mL of absolute acetonitrile, and the mixture was degassed with an argon stream and irradiated with argon continuously

flowing for ~2.5 h (until the orange-yellow colour of CAN had disappeared). The reaction mixture was concentrated under vacuum, the residue dissolved with water and extracted with ethyl acetate. The combined organic fractions were dried ($MgSO_4$) and the solvent removed under vacuum.

^{1}H NMR analysis of the reaction mixture was performed after addition of a known amount of the standard benzene to the crude reaction mixture.

The spectroscopical data of the products formed in the reactions of $NO_3^\bullet$ with **1a** and **1b**, respectively, were obtained from independent experiments through isolation and purification of the compounds by column chromatography (SiO_2, ethyl acetate) or HPLC.

1. Reaction of $NO_3^\bullet$ with 3′,5′-di-O-methyl-N-methylthymidine (**1a**)

*(a) 5-Nitratomethylene-3′,5′-di-O-methyl-N-methyl-2′-desoxyuridine (**2a**)* (could not be obtained without impurities from aldehyde **4a**). δ_H ($CDCl_3$, 300 MHz) 8.20 (s, 1H), 6.30 (dd, *J* 6.0, 6.0 Hz, 1H), 5.29 (dd, *J* 12.5, 0.7 Hz, 1H), 5.21 (dd, *J* 12.5, 0.7 Hz, 1H), the further upfield signals could not be separated from those of **4a**. *m/z* (CI) 346 (19) [M^++H], 299 (100), 283 (32), 155 (92), 145 (80). *m/z* (HR-EI). Calc. for $C_{13}H_{19}N_3O_8$: 345.11722. Found: 345.11690.

*(b) 5-Hydroxymethylene-3′,5′-di-O-methyl-N-methyl-2′-desoxyuridine (**3a**)*. $R_F = 0.16$ (SiO_2, ethyl acetate): λ_{max}/nm (CH_3CN) (lg ε) 263 (4.08), 207 (4.01). δ_H ($CDCl_3$, 500 MHz) 7.88 (s, 1H), 6.32 (dd, *J* 7.3, 6.1 Hz, 1H), 4.47 (dd, *J* 12.9, 0.8 Hz, 1H), 4.35 (dd, *J* 12.9, 0.6 Hz, 1H), 4.14 (dd, *J* 5.3, 2.6 Hz, 1H), 4.01 (dt, *J* 5.9, 2.9 Hz, 1H), 3.68 (dd, *J* 10.6, 2.7 Hz, 1H), 3.56 (dd, *J* 10.6, 2.7 Hz, 1H), 3.43 (s, 3H), 3.35 (s, 3H), 3.34 (s, 3H), 2.45 (ddd, *J* 13.7, 6.1, 2.9 Hz), 2.06 (ddd, *J* 13.5, 7.3, 6.0 Hz, 1H). δ_C ($CDCl_3$, 125 MHz) 163.7, 150.9, 135.5, 112.7, 86.4, 84.1, 81.1, 73.0, 60.2, 59.3, 57.1, 37.9, 27.8. *m/z* (EI) 300 (11%, M^+), 145 (100). *m/z* (HR-EI). Calc. for $C_{13}H_{20}N_2O_6$: 300.13214. Found: 300.13200. *m/z* (HR-ESI). Calc. for $C_{13}H_{20}N_2O_6$+Na: 323.1219. Found: 323.1220. Calc. for $C_{12}{}^{13}CH_{20}N_2O_6$+Na: 324.1219. Found: 324.1239.

*(c) 5-Formyl-3′,5′-di-O-methyl-N-methyl-2′-desoxyuridine (**4a**)*. λ_{max}/nm (CH_3CN) (lg ε) 292 (3.17). δ_H ($CDCl_3$, 500 MHz) 9.97 (s, 1H), 8.81 (s, 1H), 6.22 (t, *J* 6.5 Hz, 1H), 4.19 (q, *J* 2.3 Hz, 1H), 3.99 (dt, *J* 5.6, 2.7 Hz, 1H), 3.67 (dd, *J* 10.6, 2.5 Hz, 1H), 3.54 (dd, *J* 10.6, 2.2 Hz, 1H), 3.41 (s, 3H), 3.30 (s, 6H), 2.53 (ddd, *J* 13.8, 6.2, 2.9 Hz, 1H), 2.08 (m, 1H). δ_C ($CDCl_3$, 125 MHz) 187.0, 161.9, 150.2, 144.2, 110.3, 87.8, 84.9, 81.2, 72.6, 59.3, 56.9, 38.8, 27.6. *m/z* (EI) 298 (13, M^+), 221 (26), 145 (100). *m/z* (HR-EI). Calc. for $C_{13}H_{18}N_2O_6$: 298.11649. Found: 298.11630. *m/z* (HR-ESI). Calc. for $C_{13}H_{18}N_2O_6$+H: 299.1243. Found: 299.1247. Calc. for $C_{12}{}^{13}CH_{18}N_2O_6$+H: 300.1277. Found: 300.1267.

*(d) 5-Carboxy-3′,5′-di-O-methyl-N-methyl-2′-desoxyuridine (**5a**)*. (NB independently synthesized to confirm spectroscopic data): 10.34 mg (36.41 µmol) **1a** and 40.42 mg (73.73 µmol) CAN were dissolved in 2 mL of abs. acetonitrile and irradiated under a continuous argon flow for 3 min at $\lambda \geq 280$ nm in a quartz cuvette. For spectroscopical characterization the reaction product was isolated by semi-preparative HPLC directly from the reaction mixture.

UV (CH_3CN); λ_{max}/nm (lg ε) 279 (3.92), 218 (3.99). δ_H ($CDCl_3$, 600 MHz) 9.21 (s, 1H), 6.29 (t, *J* 6.5 Hz, 1H), 4.26 (dd, *J* 2.3, 2.3 Hz, 1H), 4.05 (dt, *J* 5.8, 2.7 Hz, 1H), 3.75 (dd, *J* 10.6, 2.5 Hz, 1H), 3.60 (dd, *J* 10.6, 2.1 Hz, 1H), 3.48 (s, 3H), 3.41 (s, 3H), 3.36 (s, 3H), 2.98 (br, 1H), 2.60 (ddd, *J* 13.8, 6.2, 2.7 Hz, 1H), 2.15 (ddd, *J* 13.9, 6.8, 5.8 Hz, 1H). δ_C ($CDCl_3$, 150.9 MHz) 165.1, 163.3, 149.5, 147.3, 101.6, 88.2, 85.1, 81.3, 72.6, 59.5, 56.9, 38.8, 28.2. *m/z* (EI) 314 (4, M^+), 145 (100), 126 (8), 11 (40). *m/z* (CI) 315 (34, M^++H), 171 (79), 145 (100), 113 (58). *m/z* (HR-EI). Calc. for $C_{13}H_{18}N_2O_7$: 314.11139. Found: 314.11110. Calc. for $C_{12}{}^{13}CH_{18}N_2O_7$: 315.11475. Found: 315.11450.

*(e) 3′,5′-Di-O-methyl-N-methyl-2′-desoxyuridine (**6a**)*.[49] δ_H ($CDCl_3$, 500 MHz) 7.72 (d, *J* 8.1 Hz, 1H), 6.28 (dd, *J* 7.6, 6.0 Hz, 1H), 5.77 (d, *J* 8.1 Hz, 1H), 4.15 (dd, *J* 5.6, 2.8 Hz, 1H), 3.99 (ddd, *J* 6.0, 2.7, 2.7 Hz, 1H), 3.66 (dd, *J* 10.5, 2.8 Hz, 1H), 3.55 (dd, *J* 10.5, 2.9 Hz, 1H), 3.42 (s, 3H), 3.35 (s, 3H), 3.32 (s, 3H), 2.47 (ddd, *J* 13.7, 6.0, 2.6 Hz, 1H), 2.03 (ddd, *J* 13.6, 7.6, 6.0 Hz, 1H). δ_C ($CDCl_3$, 125 MHz) 163.0, 151.1, 137.7, 101.7, 86.3, 83.8, 81.2, 72.9, 59.3, 56.9, 37.7, 27.6.

(f) 5-Acetamidomethyl-3′,5′-di-O-methyl-N-methyl-2′-desoxyuridine (7a). δ_H ($CDCl_3$, 500 MHz) 8.01 (s, 1H), 6.58 (s, 1H), 6.32 (dd, *J* 7.5, 6.1 Hz, 1H), 4.16 (m, 2H), 4.09 (dd, *J* 14.3, 5.7 Hz, 1H), 4.01 (dt, *J* 5.5, 2.5 Hz, 1H), 3.69 (dd, *J* 10.5, 2.8 Hz, 1H), 3.58 (dd, *J* 10.5, 2.8 Hz, 1H), 3.49 (s, 3H), 3.35 (s, 3H), 3.34 (s, 3H), 2.44 (ddd, *J* 13.8, 6.0, 2.6 Hz, 1H), 2.08 (m, 1H), 1.99 (s, 3H). δ_C ($CDCl_3$, 125 MHz) 170.8, 163.7, 150.6, 137.2, 109.7, 86.4, 84.0, 81.3, 72.9, 59.2, 56.9, 37.7, 37.6, 27.7, 23.2. *m/z* (HR-ESI). Calc. for $C_{15}H_{23}N_3O_6$+H: 342.1665. Found: 342.1667. Calc. for $C_{14}{}^{13}CH_{23}N_3O_6$+H: 343.1699. Found: 343.1694.

*(g) 5-(N-Nitroso)acetamidomethyl-3′,5′-di-O-methyl-N-methyl-2′-desoxyuridine (**8a**)*. δ_H ($CDCl_3$, 500 MHz) 7.80 (s, 1H), 6.27 (dd, *J* 7.6, 6.0 Hz, 1H), 4.77 (d, *J* 14.6 Hz, 1H), 4.58 (d, *J* 14.6 Hz, 1H), 4.18 (q, *J* 2.7 Hz, 1H), 4.01 (dt, *J* 5.2, 2.5 Hz, 1H), 3.74 (dd, *J* 10.6, 2.7 Hz, 1H), 3.58 (dd, *J* 10.6, 2.7 Hz, 1H), 3.49 (s, 3H), 3.36 (s, 3H), 3.28 (s, 3H), 2.79 (s, 3H), 2.46 (ddd, *J* 13.7, 6.0, 2.5 Hz, 1H), 2.04 (m, 1H). δ_C ($CDCl_3$, 125 MHz) 174.0, 162.0, 150.7, 138.4, 106.5, 86.6, 84.2, 81.5, 73.2, 59.5, 57.0, 37.9, 35.6, 27.9, 22.8. *m/z* (HR-ESI). Calc. for $C_{15}H_{22}N_4O_7$+H: 371.1567. Found: 371.1568. Calc. for $C_{14}{}^{13}CH_{22}N_4O_7$+H: 372.1600. Found: 372.1599.

2. Reaction of $NO_3^\bullet$ with 3′,5′-di-O-Acetylthymidine (**1b**)

*(a) 5-Nitratomethylene-3′,5′-di-O-acetyl-2′-desoxyuridine (**2b**)*. λ_{max}/nm (CH_3CN) (lg ε) 264 (4.08), 205 (4.17). δ_H ($CDCl_3$, 300 MHz) 9.35 (br, 1H), 7.81 (d, *J* 0.7 Hz, 1H), 6.26 (dd, *J* 5.1, 8.6 Hz, 1H), 5.29 (dd, *J* 0.7, 12.6 Hz, 1H), 5.22 (dt, *J* 2.0, 4.3 Hz, 1H), 5.21 (dd, *J* 0.7, 12.6 Hz, 1H), 4.45 (dd, *J* 5.1, 12.7 Hz, 1H), 4.31 (m, 1H), 4.31 (dd, *J* 3.0, 12.7 Hz, 1H), 2.59 (ddd, *J* 1.8, 5.5, 14.3 Hz, 1H), 2.13 (s, 3H), 2.12 (s, 3H), 2.12 (m, 1H). δ_C ($CDCl_3$, 75.5 MHz) 170.5, 170.4, 161.8, 149.7, 141.3, 106.7, 85.8, 82.9, 74.1, 67.0, 63.7, 38.2, 20.9, 20.8. *m/z* (EI) 387 (<1, M^+), 201 (15), 81 (100). *m/z* (CI) 388 (3, M^++H), 341 (7), 325 (8), 231 (37), 81 (100).

*(b) 5-Hydroxymethylene-3′,5′-di-O-acetyl-2′-desoxyuridine (**3b**)* (NB independently synthesized to confirm spectroscopic data): 50 mg (0.13 mmol) **2b** and 32 mg (0.19 mmol) 4-toluene sulfonic acid hydrate were dissolved in 15 mL of THF and stirred for 15 h at room temperature. The reaction mixture was quenched with saturated aqueous sodium bicarbonate and filtered (SiO_2, ethyl acetate). Column chromatography (SiO_2, ethyl acetate) yielded 7 mg (16%, $R_F = 0.10$) **3b** as a colourless oil.

δ_H ($CDCl_3$, 500 MHz) 9.42 (s, 1H), 7.59 (s, 1H), 6.31 (dd, *J* 8.4, 5.6 Hz, 1H), 5.22 (dt, *J* 8.4, 5.6 Hz, 1H), 4.47 (d, *J* 13.5 Hz,

1H), 4.39 (m, 2H), 4.32 (dd, *J* 12.2, 3.1 Hz, 1H), 4.27 (dt, *J* 4.2, 2.9 Hz, 1H), 2.51 (ddd, *J* 14.2, 5.6, 1.7 Hz, 1H), 2.20 (m, 1H), 2.13 (dd, *J* Hz, 3H), 2.11 (dd, *J* Hz, 3H). δ_C ($CDCl_3$, 125 MHz) 170.8, 170.6, 163.5, 150.2, 136.6, 114.6, 85.5, 82.6, 74.3, 64.0, 58.4, 37.9, 21.0, 20.9. *m/z* (HR-ESI). Calc. for $C_{14}H_{18}N_2O_8$+Na: 365.0961. Found: 365.0963. Calc. for $C_{13}{}^{13}CH_{18}N_2O_8$+Na: 366.0994. Found: 366.0992.

*(c) 5-Formyl-3′,5′-di-*O*-acetyl-2′-desoxyuridine* ***(4b)***. λ_{max}/nm (CH_3CN) (lg ε) 292 (3.49). δ_H ($CDCl_3$, 500 MHz) 10.01 (s, 1H), 9.95 (s, 1H), 8.47 (s, 1H), 6.28 (dd, *J* 7.7, 5.9 Hz, 1H), 5.23 (dt, *J* 6.3, 2.2 Hz, 1H), 4.37 (dd, *J* 11.1, 2.2 Hz, 1H), 4.32 (m, 2H), 2.59 (ddd, *J* 14.3, 5.9, 2.3 Hz, 1H), 2.26 (m, 1H), 2.16 (s, 3H), 2.09 (s, 3H). δ_C ($CDCl_3$, 125 MHz) 186.0, 170.8, 170.6, 162.3, 149.4, 144.9, 111.6, 86.3, 83.2, 74.1, 63.7, 38.7, 20.9, 20.7. *m/z* (EI) 340 (1, M^+), 201 (11), 81 (100). *m/z* (HR-ESI) Calc. for $C_{14}H_{16}N_2O_8$+H: 341.0985. Found: 341.0983. Calc. for $C_{13}{}^{13}CH_{16}N_2O_8$+H: 342.1019. Found: 342.1013.

*(d) 3′,5′-Di-*O*-acetyl-2′-desoxyuridine* ***(6b)***. δ_H ($CDCl_3$, 500 MHz) 8.47 (s, 1H), 7.53 (d, *J* 8.2 Hz, 1H), 6.26 (dd, *J* 8.2, 7.5 Hz, 1H), 5.80 (d, *J* 8.2 Hz, 1H), 4.38 (dd, *J* 12.1, 4.2 Hz, 1H), 4.32 (dd, *J* 12.1, 3.3 Hz, 1H), 4.28 (dd, *J* 6.6, 3.4 Hz, 1H), 2.55 (ddd, *J* 14.2, 5.6, 2.1 Hz, 1H), 2.17 (ddd, *J* 14.2, 7.7, 5.6 Hz, 1H), 2.12 (s, 3H), 2.11 (s, 3H). δ_C ($CDCl_3$, 125 MHz) 170.5, 170.4, 163.1, 149.9, 139.3, 102.8, 85.6, 82.6, 74.2, 63.9, 38.1, 21.0, 20.9. *m/z* (HR-ESI). Calc. for $C_{13}H_{16}N_2O_7$+H: 313.1036. Found: 313.1033. Calc. for $C_{12}{}^{13}CH_{16}N_2O_7$+H: 314.1069. Found: 314.1066.

*(e) 3′,5′-Di-*O*-acetyl-5-(*N*-nitroso)acetamidomethyl-2′-desoxyuridine* ***(8b)***. δ_H ($CDCl_3$, 500 MHz) 8.22 (s, 1H), 7.48 (s, 1H), 6.22 (dd, *J* 8.7, 5.4 Hz, 1H), 5.23 (dt, *J* 6.4, 1.8 Hz, 1H), 4.78 (d, *J* 14.8 Hz, 1H), 5.54 (d, *J* 14.8 Hz, 1H), 4.43 (dd, *J* 12.2, 4.5 Hz, 1H), 4.33 (dd, *J* 12.2, 3.1 Hz, 1H), 4.28 (m, 1H), 2.79 (s, 3H), 2.52 (ddd, *J* 14.2, 5.4, 1.5 Hz, 1H), 2.19 (s, 3H), 2.12 (m, 1H), 2.12 (s, 3H). *m/z* (HR-ESI). Calc. for $C_{16}H_{20}N_4O_9$+H: 413.1309. Found: 413.1309. Calc. for $C_{15}{}^{13}CH_{20}N_4O_9$+H: 414.1342.1600. Found: 414.1339.

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