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# The reactivity of the 2-deoxyribonolactone lesion in single-stranded DNA and its implication in reaction mechanisms of DNA damage and repair

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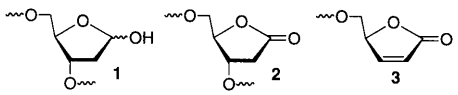
## ABSTRACT

**The formal C1'-oxidation product, 2-deoxyribonolactone, is formed as a result of DNA damage induced via a variety of agents, including γ-radiolysis and the enediyne antitumor antibiotics. This alkaline labile lesion may also be an intermediate during DNA damage induced by copper–phenanthroline. Oligonucleotides containing this lesion at a defined site were formed via aerobic photolysis of oligonucleotides containing a photolabile ketone, and were characterized by gel electrophoresis and electrospray mass spectrometry (ESI-MS). Treatment of oligonucleotides containing the lesion with secondary amines produces strand breaks consisting of 3'-phosphate termini, and products which migrate more slowly in polyacrylamide gels. MALDI-TOF mass spectrometry analysis indicates that the slower moving products are formal adducts of the β-elimination product resulting from 2-deoxyribonolactone and one molecule of amine. The addition of β-mercaptoethanol to the reaction mixture produces thiol adducts as well. The stability of these adducts suggests that they cannot be the labile species characterized by gel electrophoresis in copper–phenanthroline-mediated strand scission. The characterization of these adducts by mass spectrometry also provides, by analogy, affirmation of proposals regarding the reactivity of nucleophiles with the β-elimination product of abasic sites. Finally, the effects of this lesion and the various adducts on DNA repair enzymes are unknown, but their facile generation from oligonucleotides containing a photolabile ketone suggests that such issues could be addressed.**

## INTRODUCTION

Deglycosylated nucleotides are a ubiquitous family of lesions that are produced in DNA. Of these lesions, apyrimidic/apurinic abasic sites (**1**) and 2-deoxyribonolactone (**2**) are two prominent modifications that have been characterized. Abasic sites are produced as a result of a broad range of chemical processes which insult DNA *in vitro* and *in vivo*. These processes include alkylation and concomitant labilization of the glycosidic bond, as well as enzymatic deglycosylations (e.g. uracil deglycosylase) due to the transformation of cytosine bases during oxidative stress (1–5). Abasic sites are also formed as intermediates by a number of other repair enzymes. The effects of abasic sites (and their models) on polymerase activity, as well as DNA structure and stability, are well documented (6–9). The chemical lability of the abasic site has also been examined and even employed as a mechanistic probe of repair enzymes (10–12). In contrast, considerably less is known about the chemistry of the 2-deoxyribonolactone lesion (**2**) in DNA. However, **2** has recently been independently generated at defined sites in oligonucleotides from two different photochemical precursors (13–16). Herein, we wish to report initial studies on the reactivity of **2** in oligonucleotides, and the possible implications of the observed transformations of this lesion to related chemical and enzymatic processes.




2-Deoxyribonolactone (**2**) is believed to be produced upon reaction of DNA with a variety of naturally occurring and unnatural damaging agents (17–21). The lactone may also be formed transiently during copper–phenanthroline-induced DNA strand scission (22). Attack of DNA by these damaging agents may yield **2** via the respective radical, followed by further oxidation. Alternatively, **2** may also result from direct two-electron oxidation, or initial one-electron oxidation of the pendant nucleobase (23–25). While in some instances **2** has been chemically characterized, in many instances its presence is discerned in a more indirect manner, such as via product deuterium isotope effects and via the general formation of alkaline labile lesions (26–28). The alkaline labile nature of **2** is typically revealed under stringent conditions (aqueous piperidine, 90–95°C), which releases fragments of DNA containing 5'- and 3'-phosphate termini, presumably via β,δ-elimination. The β-elimination product (**3**) has been proposed as a labile product of direct strand scission during DNA damage mediated by copper–phenanthroline and a transition metal complex (23,29). This structural assignment is based upon identification of a labile product by polyacrylamide gel electrophoresis (PAGE) which appears to be readily transformed into a 3'-phosphate-containing

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fragment. No spectroscopic or other corroborative evidence in support of this assignment has been reported.

## MATERIALS AND METHODS

### General procedures

Oligodeoxyribonucleotide synthesis (**6** and **7**) was carried out on an Applied Biosystems Incorporated 380B DNA synthesizer using standard protocols as described previously (13,15). The purified oligonucleotides were characterized by electrospray mass spectrometry (ESI-MS) (15). Phosphoramidites and all other oligonucleotide synthesis reagents were obtained from Glen Research. DNA manipulation, including enzymatic labeling, was carried out using standard procedures (30). Oligonucleotides were partially sequenced using a reaction specific for adenine (31). Electrospray and MALDI-TOF mass spectrometry samples were prepared by ethanol precipitating from $NH_4OAc$. Electrospray mass spectra were obtained on a Fisons VG Quattro. MALDI-TOF mass spectra were obtained on a PerSeptive Biosystems Voyager-DE RP. Preparative and analytical oligonucleotide separations were carried out on 20% polyacrylamide denaturing gel [5% crosslink, 45% urea (w/w)]. T4 polynucleotide kinase was obtained from New England Biolabs. [$\gamma$-$^{32}$P]ATP was purchased from Amersham Pharmacia Biotech. Radioactive samples were counted by Cerenkov counting, using a Packard Tri-Carb 1500 scintillation counter. Quantification of radiolabeled oligonucleotides was carried out using a Molecular Dynamics Phosphorimager equipped with ImageQuant Version 3.3 software. All photolyses of oligonucleotides were carried out in Pyrex tubes (0.25 inch i.d.) using a Rayonet Photoreactor (RPR-100) equipped with 16 lamps having a maximum output at 350 nm.

### Mass spectral analysis of photolysates

Prior to photolysis, oligonucleotide **6** was precipitated (twice) from ammonium acetate. The oligonucleotide (1 nmol) and β-mercaptoethanol (0.1 mM) in water (300 µl) were photolyzed for 1 h under aerobic conditions. The crude photolysate was transferred to an eppendorf tube, and the photolysis tube was washed with $H_2O$ (2 × 50 µl). The photolysate was appropriately apportioned, and treated with either *N*,*N*'-dimethylethylenediamine (**9**; 5 or 100 mM) at 37°C for 20 min, or piperidine (100 mM) at 37 or 90°C for 20 min and then dried. Reactions carried out in the presence of β-mercaptoethanol contained either 5 or 100 mM of the thiol.

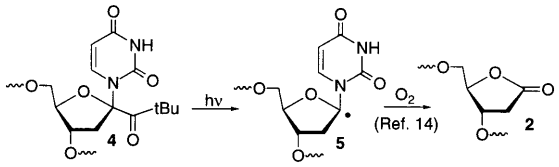
### Gel electrophoresis analysis of photolysates

Radiolabeled oligonucleotide (**7**) in 10 mM phosphate (pH 7.2) and 100 mM NaCl was photolyzed for 20 min under aerobic conditions. The photolyzed solution was transferred to an Eppendorf tube, and the photolysis tube was washed with $H_2O$ (2 × 50 µl). The photolysate was appropriately apportioned, and treated with either *N*,*N*'-dimethylethylenediamine (5 or 100 mM) at 37°C for 20 min, or piperidine (100 mM) at 37 or 90°C for 20 min, and then precipitated with 0.3 M NaOAc. Reactions carried out in the presence of β-mercaptoethanol contained either 5 or 100 mM of the thiol. The samples were resuspended in formamide loading buffer at 55°C for 5 min with intermittent vortexing. Note, care must be taken when resuspending the samples. Prolonged heating will induce strand scission at sites containing 2-deoxyribonolactone (**2**). Analysis of radiolabeled oligonucleotides separated by gel electrophoresis was carried out using a Molecular Dynamics Phosphorimager.

### Preparation of DMT–furanone adduct (18)

*N*,*N*-dimethylethylenediamine (0.66 g, 7.52 mmol) was added to a solution of the lactone (**17**; 0.15 g, 0.37 mmol) in $CH_3CN$:$H_2O$ [2:1 (v/v), 12 ml] at 37°C. After 1 h, the reaction mixture was concentrated *in vacuo*. Column chromatography (2:1 hexanes:EtOAc to 5:5:1 hexanes:EtOAc:MeOH) yielded **18** as a white foam (0.11 g, 56%). m.p. 57–64°C; $^1$H NMR (300 MHz, $CDCl_3$) δ 7.45 (d, 2H, J. = 7.2 Hz), 7.37–7.18 (m, 7H), 6.86–6.81 (m, 4H) apparent d, 3.98–3.87 (m, 1H), 3.80 (s, 6H), 3.50 (dd, 1H, J. = 6.9, 15.3 Hz), 3.33–3.20 (m, 3H), 2.96 (s, 3H), 2.97–2.90 (m, 1H), 2.75–2.57 (m, 5H), 2.38 (s, 3H); $^{13}$C NMR (75 MHz, $CDCl_3$) δ 174.0, 158.7, 145.2, 136.40, 136.35, 130.3, 128.4, 128.0, 126.9, 113.3, 86.5, 70.3, 65.7, 60.4, 55.4, 52.9, 47.4, 41.5, 35.9, 34.7; IR (film) 3399 (br), 3058, 2932, 2836, 1634, 1608, 1509, 1463, 1445, 1398, 1301, 1250, 1177, 1069, 1034, 911, 829, 728 cm$^{-1}$. HRMS (FAB) calculated 505.2704 (M + H), found 505.2691.

Scheme 1




## RESULTS AND DISCUSSION

Recently, we showed that aerobic photolysis of chemically synthesized oligodeoxynucleotides containing **4** incorporated at a defined site produces alkaline labile lesions which are consistent with the formation of **2** via the intermediacy of **5** (Scheme 1) (13,15). This proposal is corroborated by product studies on monomeric **4** which produced **2** in ~90% yield upon photolysis under aerobic conditions (14). In this study, the reactivity of **2** has been examined via two complementary techniques, mass spectrometry and gel electrophoresis, using two appropriate oligonucleotides. Mass spectrometry was carried out on the

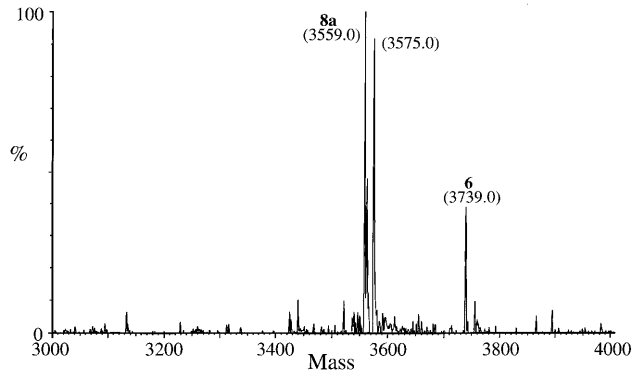



**Figure 1.** Electrospray mass spectrum of the crude photolysate of **6**. Calculated mass: **6**, 3738.7; **8a**, 3558.3.

dodecamer (**6**), which could be obtained in quantities suitable for mass spectrometric analysis. A 30mer (**7**) was employed in experiments involving gel-electrophoretic analysis, where radiolabeling facilitated the use of subpicomole amounts of material. We have now obtained direct evidence for the formation of **2** in an oligonucleotide by ESI-MS upon photolysis of **6** in $H_2O$ (**8a**; Fig. 1). Under the conditions of the ESI-MS experiment, one observes **2** and a product containing 16 additional atomic mass units (3575.0 a.m.u.). The structure of this latter product is unknown at this time. However, ammonia or water addition products of **8a** (produced via reaction of **2** with the ammonium acetate buffer used in the ESI-MS experiment), as well as a higher oxidation product of the oligonucleotide containing **2** are possible explanations for this unexpected species. Similar attempts at characterizing **2** using MALDI-TOF mass spectrometry only revealed fragmentation products containing phosphate termini (e.g. **10a**).

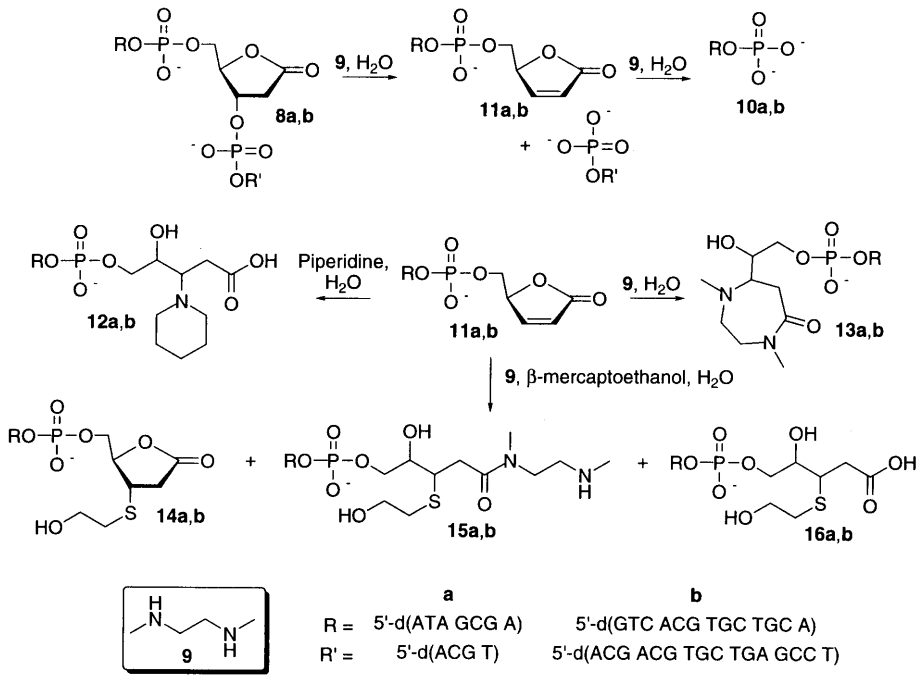
5'-d(ATA GCG A4A CGT)
**6**

5'-d(GTC ACG TGC TGC A4A CGA CGT GCT GAG CCT)
**7**

Having ascertained that **4** produces **2** in an oligonucleotide upon irradiation in the presence of $O_2$, we attempted to use this labile lesion as a precursor for **3**. A variety of amines and small peptides have been utilized for inducing β-elimination from abasic sites (10,32). In many instances, the initial elimination product was not observed, but has been trapped by thiols and possibly amines (see below). Recently, it was reported that a 0.1 M buffered (pH 7.4) solution of *N*,*N*'-dimethylethylenediamine (**9**) produces the β-elimination product of an abasic site (**1**) at 37°C (32). Consequently, the diamine (**9**) appeared to be particularly attractive for generating the β-elimination product (**3**) derived from **2** in an oligonucleotide (**7**) containing the photolabile ketone (**4**). We found that the product distribution, as analyzed by gel electrophoresis, was comparable whether buffered (100 mM) or unbuffered (5 mM) solutions of **9** were employed (data not shown).

In either case, the previously characterized 3'-phosphate product (**10b**), resulting from β,δ-elimination, was observed as the major product (Fig. 2) (13). However, small amounts of a more slowly moving product were also observed. This product, or one which migrates at the same rate in the denaturing PAGE experiment, was also observed upon treatment of the photolyzed oligonucleotide with piperidine at 90°C. Treatment of photolyzed **7** with **9** (0.1 M), or piperidine (0.1 M), at 37°C resulted in a larger amount of the respective slower moving product(s) relative to **10b** (Fig. 3). The gel mobility of this product (or products) is consistent with that previously ascribed to oligonucleotide fragments containing **3** (29). However, the stability of this strand scission product, as implied by its observation following alkaline treatment at 90°C, is inconsistent with that of the product previously observed in copper–phenanthroline-mediated cleavage experiments. Indeed, MALDI-TOF mass spectrometric analysis of the amine-treated photolysates of **6** failed to reveal the presence of **11a** (Table 1; Fig. 4). Instead, this analysis indicated that the slower moving strand scission products resulted from addition of the respective amine to the β-elimination cleavage product (e.g. **12a**, **13a**) (Table 1; Fig. 4). The adduct derived from piperidine-induced cleavage was observed as the hydrate (**12a**), whereas the product from **9** corresponded to the formal 1:1 adduct of the amine and **11a** (**13a**). The observed adducts are proposed to arise via initial conjugate addition of the amine to **11a** (Scheme 2). When *N*,*N*'-dimethylethylenediamine (**9**) is employed, subsequent intramolecular cyclization occurs to yield the observed lactam

**Scheme 2**



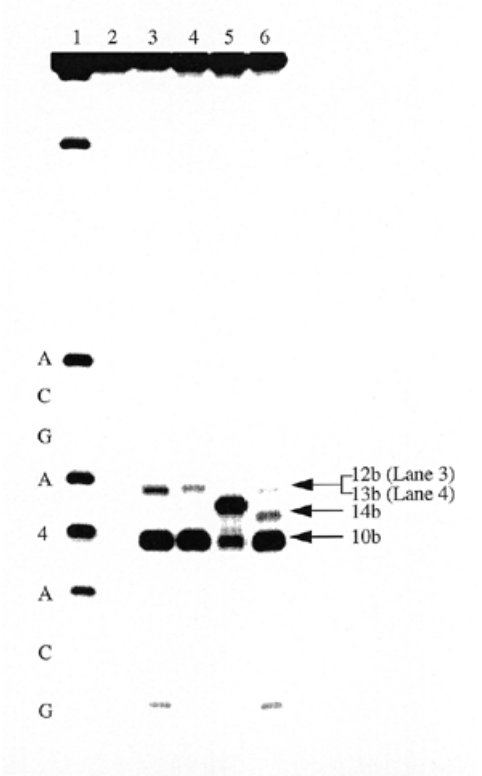



**Figure 2.** Autoradiogram of the photolysis of 5'-$^{32}$P-**7**. Lane 1, dA selective sequencing reaction. Lanes 2–6 contain photolyzed samples of 5'-$^{32}$P-**7**: lane 2, no further treatment; lane 3, piperidine (100 mM), 20 min, 90°C; lane 4, **9** (5 mM), 20 min, 37°C; lane 5, **9** (5 mM), β-mercaptoethanol (5 mM), 20 min, 37°C; lane 6, lane 5 followed by piperidine treatment as in lane 3. The faster moving product observed in lanes 3–6 is **10b**. The slower moving products observed in lanes 3–6 are **12b**, **13b**, **14b** and residual **14b** respectively.

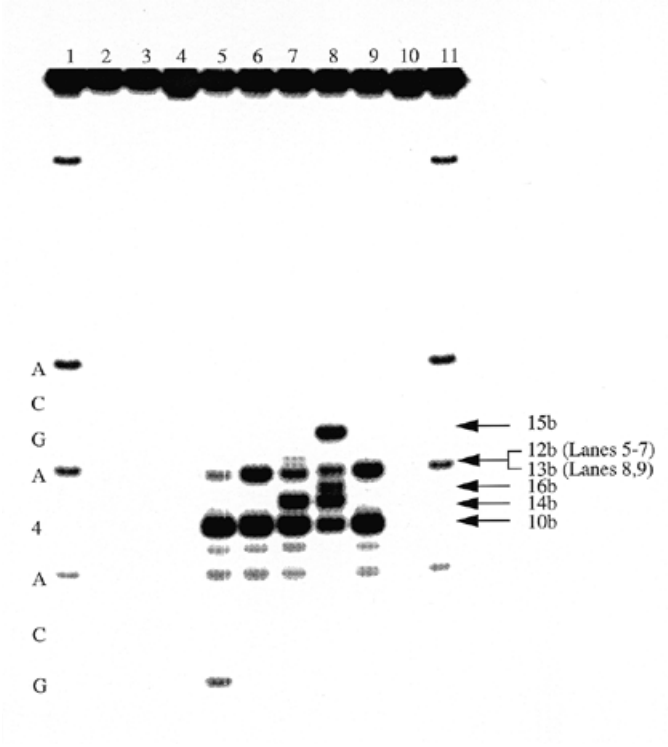


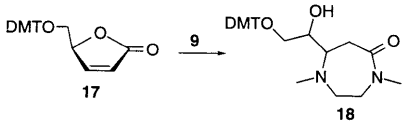

**Figure 3.** Autoradiogram of the photolysis of 5'-$^{32}$P-**7**. Lanes 1 and 11, dA selective sequencing reaction; lane 2, non-photolyzed; lane 3, non-photolyzed + piperidine (0.1 M), 30 min, 90°C; lanes 4–10 contain photolyzed samples of 5'-$^{32}$P-**7**: lane 4, no further treatment; lane 5, piperidine (100 mM), 20 min, 90°C; lane 6, piperidine (100 mM), 20 min, 37°C; lane 7, piperidine (100 mM), β-mercaptoethanol (50 mM), 20 min, 37°C; lane 8, **9** (100 mM), β-mercaptoethanol (50 mM), 20 min, 37°C; lane 9, **9** (100 mM), 20 min, 37°C; lane 10, β-mercaptoethanol (50 mM), 20 min, 37°C. The faster moving product observed in lanes 5, 6 and 9 is **10b**. The slower moving products in lanes 5, 6 and 9 are **12b**, **12b** and **13b** respectively. The products observed in lane 7 (fastest to slowest) are **10b**, **14b** and **12b**; and in lane 8: **10b**, **14b**, **16b**, **13b** and **15b**.

**Table 1.** Calculated and observed masses of oligonucleotides

| Product | Calculated mass | Observed mass |
|---|---|---|
| **6** | 3738.7 | 3739.0 (Fig. 1) |
| **8a** | 3558.3 | 3559.0 (Fig. 1) |
| **10a** | 2208.4 | 2209.4 (Figs 4 and 5); 2209.1 (Fig. 6) |
| **11a** | 2305.5 | Not observed |
| **12a** | 2408.7 | 2408.6 (Fig. 4) |
| **13a** | 2393.7 | 2393.1 (Fig. 4); 2393.0 (Fig. 6) |
| **14a** | 2383.7 | 2383.5 (Fig. 5); 2381.1 (Fig. 6) |
| **15a** | 2471.8 | 2471.7 (Fig. 5); 2471.3 (Fig. 6) |
| **16a** | 2401.7 | 2403.2 (Fig. 5); 2401.2 (Fig. 6) |

product (**13a**). Reaction of **17** with **9**, which forms **18**, supports this latter proposal (Scheme 3). In contrast, treatment with the monoamine piperidine ultimately results in hydrolysis of the lactone (**12a**), which under the alkaline reaction conditions does not recyclize. 1,4-Conjugate addition of the amines is advocated instead of initial attack at the carbonyl, based upon the known reactivity preferences of amines with α,β-unsaturated furanones (33). Based upon these observations, we suggest that the products previously observed upon reaction of abasic sites (**1**) with amines via denaturing PAGE are not the unsaturated species, but rather amine adducts (32).

**Scheme 3**




Addition of β-mercaptoethanol (5 mM) in the presence of **9** (5 mM) gave rise to a third product of intermediate migratory aptitude (Figs 2 and 3), which MALDI-TOF indicated was consistent with a formal adduct (**14a**) of the thiol with **11a** (Table 1; Fig. 5). Observation of **14a**,**b** supports the recent proposal that prolonged exposure of crude photolysates of oligonucleotides containing **4** to kinase buffer (containing dithiothreitol) results in a similar β-elimination, followed by thiol trapping (Scheme 2) (13). Subsequent treatment of this product mixture under alkaline conditions resulted in its transformation into the 3'-phosphate-containing fragment (**10b**), consistent with the

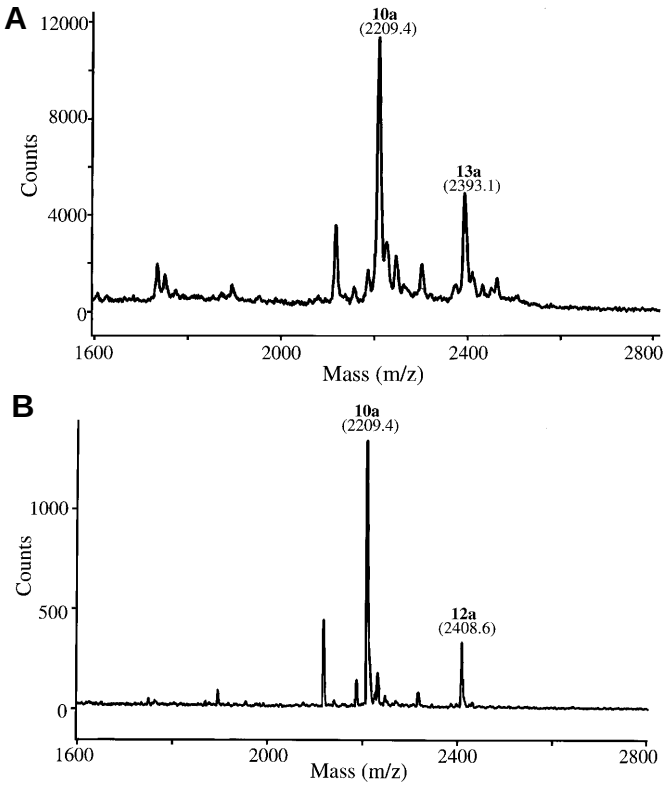



**Figure 4.** MALDI-TOF mass spectra of amine treated photolysates of **6**: (**A**) **9** (100 mM), 37°C, 20 min. For the gel electrophoretic analysis of photolyzed **7** subjected to comparable reaction conditions see Figure 3, lane 9. (**B**) Piperidine (100 mM), 37°C, 20 min. For the gel electrophoretic analysis of photolyzed **7** subjected to comparable reaction conditions see Figure 3, lane 6.

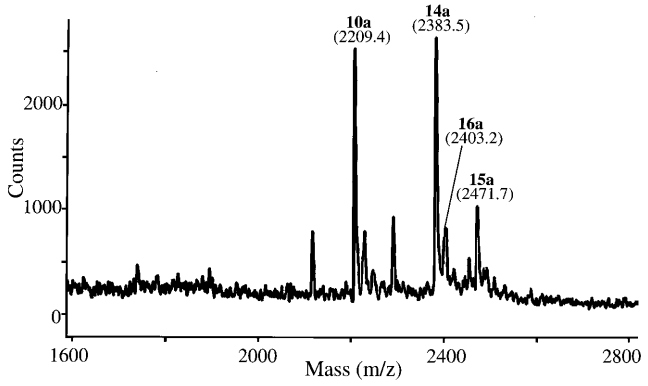



**Figure 5.** MALDI-TOF mass spectra of photolysate of **6** treated with **9** (5 mM) + β-mercaptoethanol (5 mM), 37°C, 20 min. For the gel electrophoretic analysis of photolyzed **7** subjected to comparable reaction conditions see Figure 2, lane 5.

proposed structure (Fig. 2). A minor amount of a diadduct containing one molecule each of β-mercaptoethanol and **9** (**15a**,**b**) was also detected (Table 1; Figs 3 and 5).

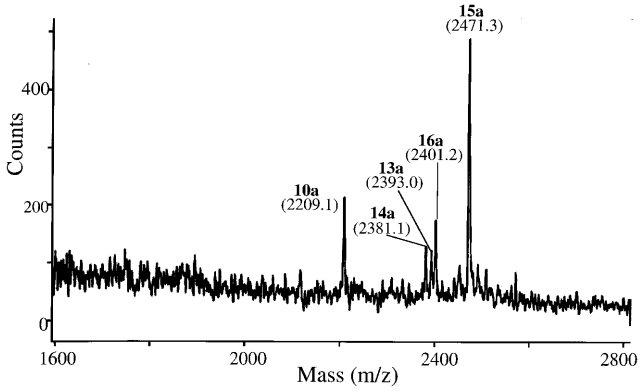



**Figure 6.** MALDI-TOF mass spectra of photolysate of **6** treated with **9** (100 mM) + β-mercaptoethanol (100 mM), 37°C, 20 min. For the gel electrophoretic analysis of photolyzed **7** subjected to comparable reaction conditions see Figure 3, lane 8.

The diadduct (**15a**,**b**) was formed in larger amounts when **2** was reacted with higher concentrations of **9** (100 mM) and thiol (100 mM) (Table 1; Figs 3 and 6). Under these more stringent conditions, a total of five cleavage products were observed by denaturing PAGE (**10b**, **13b**–**16b**) and MALDI-TOF mass spectrometry (**10a**, **13a**–**16a**). It should be noted that attempts at observing any of these products by ESI-MS were unfruitful. The failure of ESI-MS to detect any products in these experiments may be attributed to association of various amounts of the amine with the oligonucleotide molecules, which reduces the signal to noise in these experiments.

## CONCLUSIONS

These experiments demonstrate that oligonucleotides containing **2** are highly susceptible to β,δ-elimination under mild alkaline conditions. In the presence of suitable nucleophiles, such as thiols that are present at physiologically relevant concentrations (5–7 mM), the initial β-elimination product (**11a**,**b**) is trapped, presumably via a Michael reaction (Scheme 2). Thiol adducts of **3** (or its ring opened analogue) have been proposed previously in the chemistry of neocarzinostatin (34). The stability of these adducts rules them out as candidates for the metastable lesion observed by gel electrophoresis during copper–phenanthroline- and ruthenium-mediated DNA strand scission (23,29). Hence, the proposal that **3** describes the general family of metastable lesions formed by these DNA damaging agents is viable.

The observations reported above are reminiscent of those described previously for abasic sites (**1**) (10). In these earlier studies, gel electrophoresis experiments ultimately uncovered an analogous Michael reaction with thiols which helped to explain observations involving endonucleases. Similarly, the characterization of formal amine adducts of **3** suggest that the product observed in a recent report using **9** is not the β-elimination product of **1**, but rather an adduct of this species (32). The formation of amine adducts with **3** also evokes an interesting and potentially important question regarding the enzymatic repair of **2**, of which little if anything is known. If **2** is repaired by a lyase mechanism, is it possible that this lesion acts as a suicide

substrate due to covalent linking between **3** and a nucleophile present in the enzyme binding site? For this reason, as well as the inherent importance of enzymatic repair of DNA lesions, the interactions of 2-deoxyribonolactone (**2**) lesions in nucleic acids with such enzymes should be studied.

## ACKNOWLEDGEMENTS

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