The chemistry of single-stranded 4'-DNA radicals: influence of the radical precursor on anaerobic and aerobic strand cleavage

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Background: Deoxyribosylnucleotide radicals with a radical center at the 4'-position are important intermediates in radical-induced DNA strand cleavage. In the presence of O_2, these DNA radicals yield cleavage products that are partly oxidized. In the past, the postulated peroxide intermediates could not be detected directly because they were unstable under the conditions of either radical generation, the work-up procedure, or the analytical techniques used. We set out to generate and analyze these crucial intermediates in radical-induced DNA strand cleavage under mild conditions.
Results: Photolysis experiments with modified single-stranded oligonucleotides generated 4'-DNA radicals that were trapped by O_2. Using MALDI-MS, DNA peroxides could be detected directly. Depending upon the precursor, these peroxides are formed either before or after the cleavage of the single-stranded DNA radical. Reactions in the presence of $^{18}O_2$ and/or $H_2{}^{18}O$ as well as subsequent transformations to the oxidized cleavage products confirmed the structure of the DNA peroxides.
Conclusions: Our technique of selective DNA radical generation under mild conditions makes it possible to detect labile reaction products of single-stranded DNA radicals and to gain further insight into their cleavage reactions. In cases where a radical pair is formed, the shielding effect protects the DNA radical from external attack so that cleavage of the single strand competes successfully with trapping by O_2. This shielding effect might be of general importance if the DNA radicals are generated by reagents that bind to the DNA.

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Introduction

Antibiotics from the enediyne family, such as neocarzinostatin [1] and esperamycin [2], as well as metal complexes of bleomycin glycopeptides [3], induce the oxidative cleavage of DNA by generating highly reactive DNA radicals. In a similar way, chemical nucleases and drugs can generate oxidative stress by damaging DNA via radicals [4]. The research in this area is focused on the development of new selective DNA cleavers and on the elucidation of the mechanism of action of these compounds. Whereas hydroxyl radicals react rather unselectively, several agents whose active center is bound to the minor groove preferentially abstract hydrogen atoms from the 4'-and/or 5'-position of the deoxyribose [1–4]. The subsequent cleavage reactions depend on the reagent and the reaction conditions. In the presence of O_2, the glycolate **2** (Fig. 1), base propenal **3** or ketoaldehyde **4** are the typical oxidation products of the 4'-DNA radical **1**.

Recently, we have demonstrated that the 4'-deoxyribonucleotide radical **1** can be generated selectively by photolytic cleavage of the C–X bond of the modified deoxyribonucleotide shown as compound **5** in Figure 2 [5]. The formation of the reaction products was detected by MALDI-MS, which is an excellent tool for the qualitative analysis of oligonucleotides [6,7]. Kinetic experiments [8], photocurrent measurements [9] and trapping reactions [8] proved that a single-stranded 4'-DNA radical like species **1** can break the secondary C–O bond of the phosphate linkage at the 3' position in a heterolytic reaction. This leads to cleavage product **7**, which is an oligomer of lower molecular weight, and to radical cation **6**. This cation radical reacts with H_2O to generate the new 4'-DNA radical **8**. The phosphate C–O bond in the 5' position of this intermediate breaks, producing oligonucleotide **9** as the other cleavage product (Fig. 2). The strand-cleaved oligomers **7** and **9** can be described

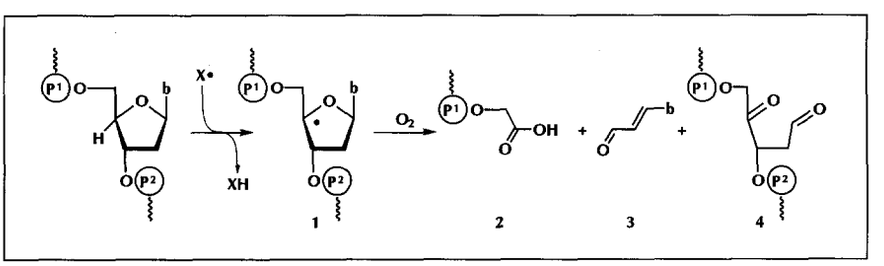

Fig. 1. Oxidative cleavage products of 4'-DNA radicals such as structure **1**, generated by bleomycin, neocarzinostatin or esperamycin. Attack by a radical (X•) causes removal of a hydrogen atom from the 4' position. Attack by molecular oxygen then leads to oxidation to a number of different products, including the glycolate shown as **2**, the base propenal **3** or the ketoaldehyde **4**.

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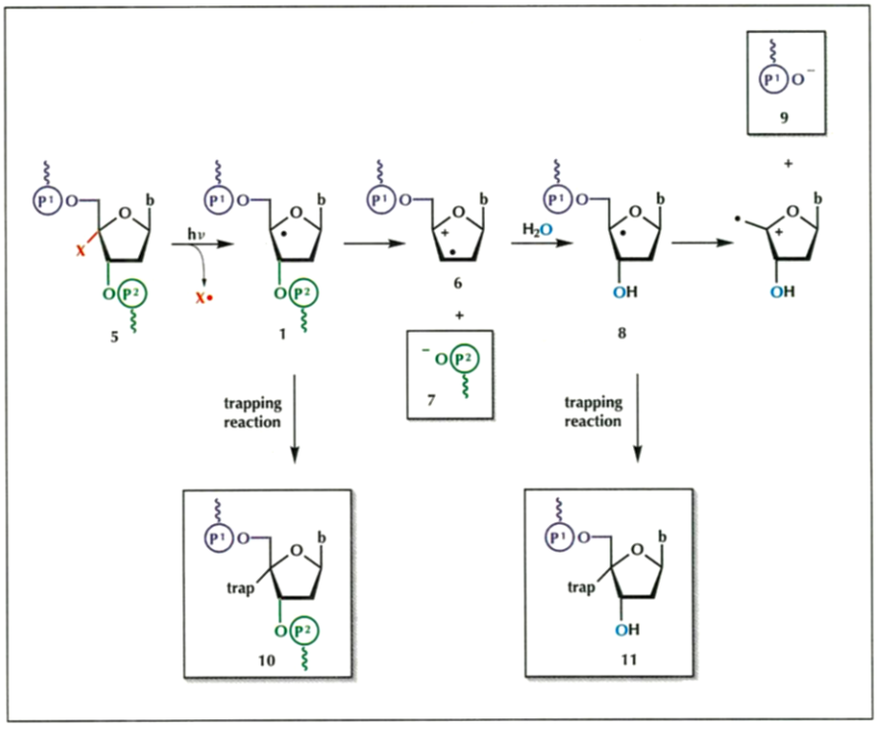

Fig. 2. Proposed mechanism for the cleavage of single-stranded 4'-DNA radicals such as structure **1** [5]. The C–O bonds of radicals **1** and **8** are cleaved heterolytically. The photoreactive leaving group X is highlighted in red. After photolysis, forming radical **1**, the C–O bond linking the deoxyribose sugar to the 3' phosphate (green) of the DNA chain undergoes heterolytic cleavage, giving compounds **6** and **7**. The cation **6** reacts with water (oxygen highlighted in light blue) to give a new radical, **8**. The C–O bond to the 5' phosphate (dark blue) then breaks, completing the DNA cleavage reaction by generating the second product, **9**. Either of the radical intermediates **1** and **8** can be trapped, for example with glutathione. hν denotes energy derived from light.

as hydrolysis products of radicals **1** and **8** where H_2O has attacked, presumably in a S_N1 substitution reaction [5,10], the carbon atoms of the deoxyribose ring and not the phosphorous atoms of the phosphate linkage. The existence of radicals **1** and **8** as intermediates has been confirmed by trapping reactions with glutathione [5].

In the radical-induced DNA strand cleavage reaction the cleaving agent and the reaction conditions both influence the formation of product [1–4]. Therefore, we examined whether the nature of group X on the DNA radical precursor **5** also has an effect on product formation. Two different radical precursors, **12** and **15**, were used. We assumed that photolysis of the phenylselenated oligonucleotide **12** should generate a radical pair in a solvent cage, because the diffusion of the hydrophobic phenylselenyl radical **13** into water should be slow (Fig. 3a). As a consequence, the intermolecular reactions of

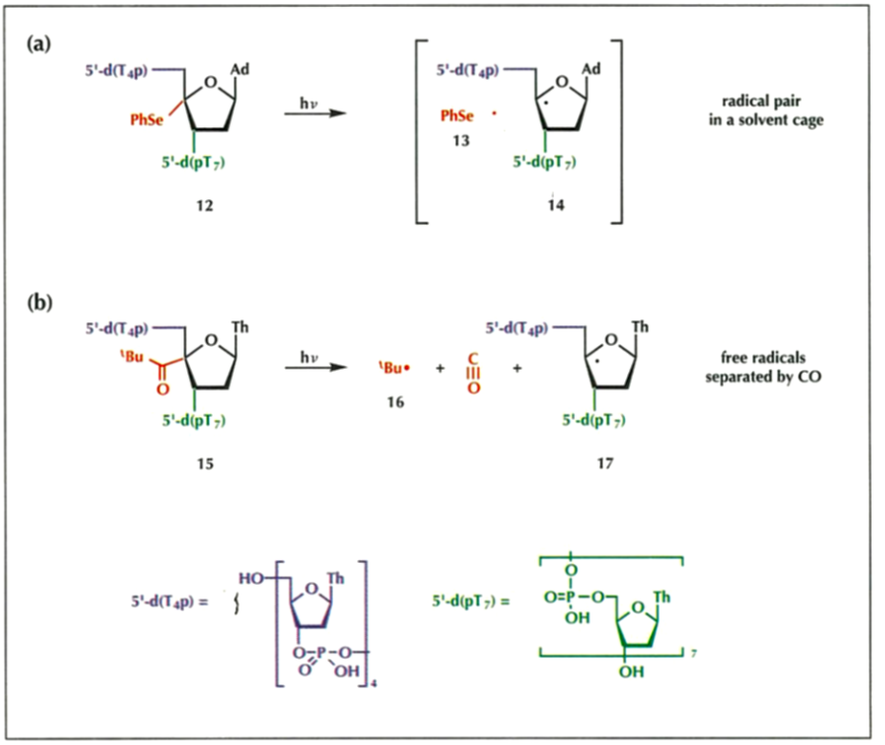

Fig. 3. Assessment of the differences between two photolytically cleavable groups, phenylselenyl (PhSe) and *tert*-butyl ketone. **(a)** Photolysis of precursor **12** results in formation of the single-stranded 4'-DNA radical **14**, next to the radical PhSe, in a solvent cage. **(b)** Photolysis of precursor **15**, however, leads to the formation of a single-stranded 4'-DNA radical **17** separated from the *tert*-butyl radical by the molecule of carbon monoxide that is also generated in the reaction. The 5'- and 3'-DNA chains are color-coded as in Fig. 2, with structures shown at the bottom of the figure.

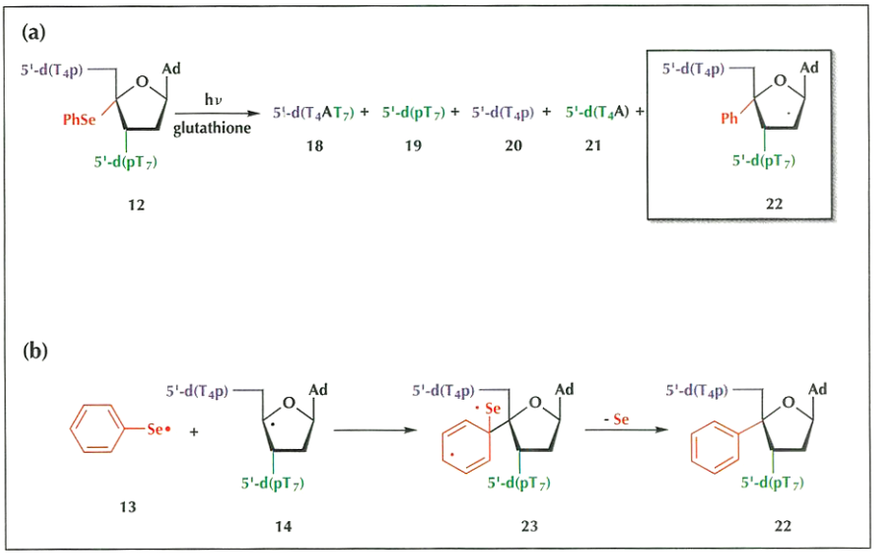

Fig. 4. Photolytic cleavage of the PhSe precursor **12** gives rise to unexpected products. **(a)** Products from single-stranded 4'-DNA radical **14** (generated by photolysis of compound **12**) in the presence of glutathione as hydrogen donor. The presence of the uncleaved DNA, compound **18**, can be explained by glutathione trapping of radical **14** (compare with trapping of compound **1** to give compound **10** in Fig. 2). **(b)** Trapping competes with a second reaction in which radicals **13** and **14** presumably recombine and eliminate a selenyl radical to give the phenylated product **22**. The other products (compounds **19–21**) can also be explained by reactions analogous to those depicted in Fig. 2.

the 4'-deoxyribonucleotide radical **14** should be retarded. In contrast, ketone **15** generates a free 4'-DNA radical **17** which is separated from the *tert*-butyl radical **16** by one molecule of carbon monoxide (Fig. 3b). Thus, the 4'-deoxyribonucleotide radical **17** should be more accessible to intermolecular reactions, for example with external radical traps.

Results and Discussion

Reductive cleavage in the presence of glutathione

Recently, we have shown that photolysis of the phenylselenated single-stranded oligomer **12** in the presence of glutathione yields compound **18**, the reduction product of the intermediate 4'-DNA radical **14**, and its cleavage products **19–21** (Fig. 4a) [5]. The formation of the products can be explained by the reaction sequence shown in Figure 2. Oligonucleotide radical **14**, which is analogous to **1**, is trapped by glutathione and yields the intact oligonucleotide **18** (compound **10** in Fig. 2). In a competing reaction, radical **14** also yields a lower mass cleavage product, oligonucleotide **19** (compound **7** in Fig. 2), and a cation radical that is analogous to **6**. After addition of water, a new neutral radical is formed (compound **8** in Fig. 2) which may be trapped by glutathione to yield **21** or may be converted to the lower mass oligonucleotide **20** by cleavage of the primary phosphate C–O bond.

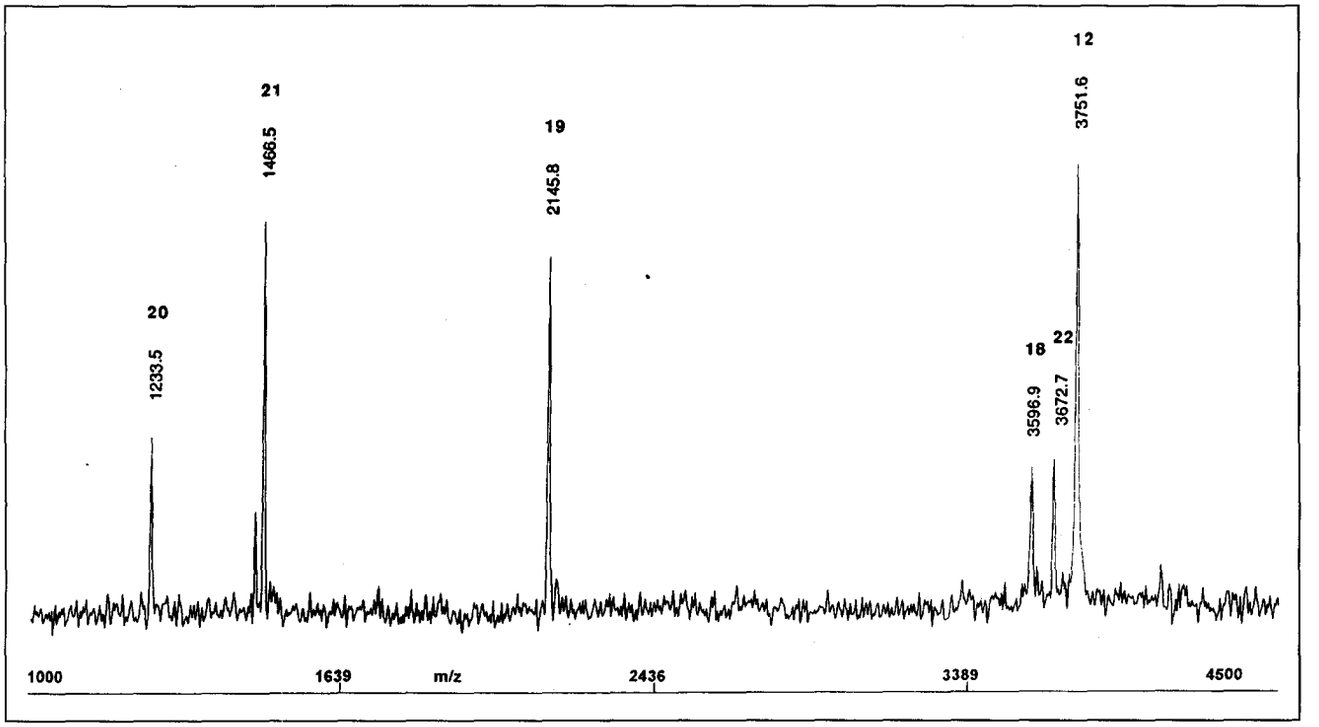

Fig. 5. MALDI-MS of the reaction products of single-stranded 4'-DNA radical **14** (generated by photolysis of compound **12**) in the presence of 0.2 mg (3.3 mM) glutathione (the formulae of the compounds are shown in Fig. 4).

We have now found that if the reaction is carried out at low glutathione concentration another compound with m/z = 3672.7 can be detected in the mass spectrum (Fig. 5). The mass is in agreement with the phenylated dodecamer **22** that could have been formed by attack of the 4'-DNA radical **14** at the *ipso* position of the phenylselenyl radical **13**. This recombination reaction leads to product **22** with biradical **23** as a possible intermediate (Fig. 4b). Cleavage reactions carried out in the presence of different amounts of glutathione are in accord with this explanation. Although a quantitative analysis of MALDI mass spectra is hardly possible [11], Figure 6a demonstrates that in the absence of glutathione the formation of the phenylated oligomer **22** is a major reaction path. In the presence of glutathione, however, the amount of reduction product **18** increases (Fig. 6b), and high glutathione concentrations suppress the formation of the phenylated oligomer **22** (Fig. 6c). Thus, glutathione can trap radicals **13** and **14** before they recombine to **23** and therefore lowers the yield of the phenylated oligomer **22**.

To explain the formation and the different amounts of the phenylated oligonucleotide **22,** we assume that the phenylselenyl radical **13** is formed as a radical pair with the 4'-DNA radical **14** in a solvent cage [12]. This is not the case if the *tert*-butyl ketone **15** is used as the radical precursor, since it generates a free DNA radical **17** and thus yields only the cleavage products **19**, **20** and 5'-$d(T_5)$ as well as the reduction product 5'-$d(T_{12})$ (Fig. 7). The formation of these products follows the reaction sequence of Figure 2, as discussed above.

In all experiments that we have carried out with ketone **15** in the presence of glutathione, the yield of the cleaved oligomer **20** was low (Fig. 7). This cleavage product is formed at the end of the reaction cascade (compound **9** in Fig. 2). Obviously, with ketone **15** as precursor, the intermediate radicals analogous to **1** and **8** are so easily accessible to glutathione that most of them are trapped before cleavage to the lower mass oligonucleotide **20** can occur.

Oxidative cleavage in the presence of O_2

Photolysis of the selenated DNA radical precursor **12** in the presence of O_2 yields the strand-cleaved oligomers **19** and **20**, and glycolate **26**. The same products are formed, although in different ratios, from ketone **15** (Fig. 8). The MALDI mass spectra showed, however, that in addition to these cleavage products, two new compounds with m/z = 1496.9 starting from compound **12**, and m/z = 3619.3 starting from compound **15** were formed (Figs 9 and 10). Both peaks increased by four mass units if the reactions were carried out in the presence of $^{18}O_2$. This demonstrates that both compounds have reacted with and incorporated O_2. However, reactions in $H_2{}^{18}O$ showed that only the oxidation product with m/z = 1496.9 has also incorporated the oxygen atom of H_2O. With this information, we assigned the structures of the peroxides as **24** (Fig. 8; m/z = 1496.9) and **25** (m/z = 3619.3). Thus, the free 4'-DNA radical **17**, generated from ketone **15**, is directly trapped by O_2, and after abstraction of hydrogen yields **25**, the peroxide of the intact DNA strand (Fig. 11).

With the selenated oligonucleotide **12** as precursor of the 4'-DNA radical, a peroxide of the intact DNA strand could not be detected by MALDI-MS (Fig. 9). A possible explanation is that radical **14** is generated together with **13** as a radical pair in a solvent cage and that the flat aromatic ring of **13** might interact with the aliphatic and/or aromatic ring systems of the oligonucleotide radical **14**. This could lead to a shielding of the radical which reduces the rate of bimolecular reactions with external traps like O_2. Monomolecular cleavage reactions of radical **14,** however, should not be much affected by formation of this pair between compounds **13** and **14**. As a result, the intramolecular cleavage of the secondary phosphate C–O

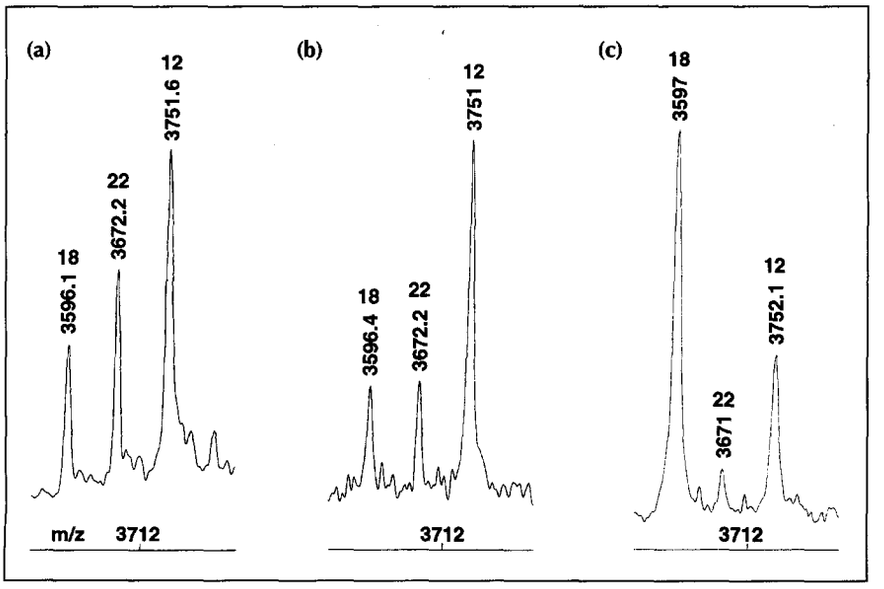

Fig. 6. MALDI-MS of radical educt **12**, reduction product **18** and phenylation product **22** (the formulae of the compounds are shown in Fig. 4). By increasing the amount of glutathione added (none in Fig. 6a, 0.2 mg (3.3 mM) in Fig. 6b, 2.0 mg (33 mM) in Fig. 6c), the ratio of product **18** to product **22** increases.

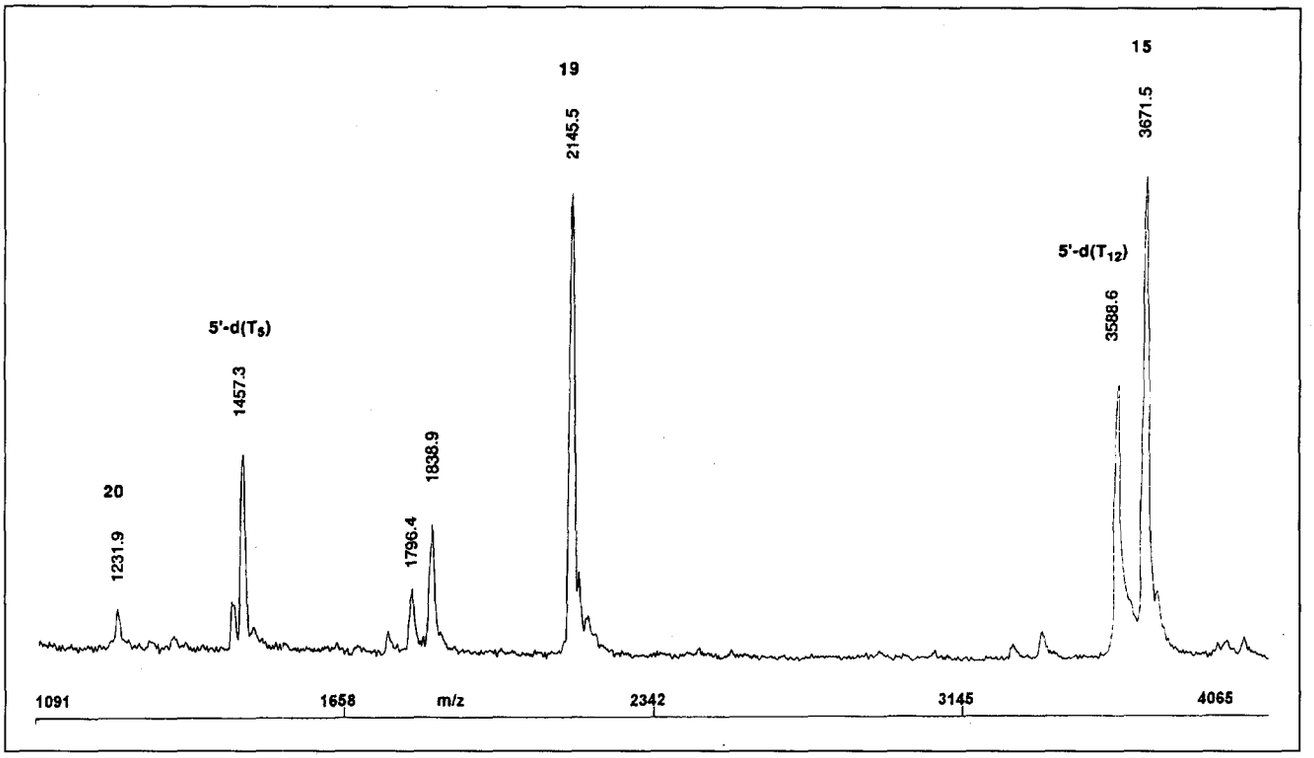

Fig. 7. MALDI-MS of the reaction products of single-stranded 4'-DNA radical **17** (generated by photolysis of compound **15**) in the presence of 0.8 mg (26 mM) glutathione (the formulae of the compounds are shown in Figs 3 and 4). The peaks with m/z = 1796.4 and 1838.9 correspond to the doubly deprotonated compounds 5'-d(T_{12}) and **15**.

bond could be faster than the intermolecular trapping reaction (Fig. 11). After addition of H_2O, this produces the 4'-oligonucleotide radical **27**. The trapping reaction by O_2 can now compete with the cleavage of the stronger primary C–O bond at the 5'-position. This leads to hydroperoxide **24**, in which the oligonucleotide strand of the precursor **12** has already been cleaved.

Our experiments demonstrate that the method used to generate the 4'-DNA radical has an important influence on the formation of hydroperoxide. Both hydroperoxides **24** and **25** seem to be precursors of glycolate **26**, however (Figs 11 and 12). We have shown recently that β-hydroxyperoxides like compound **24** easily undergo a base-catalyzed Grob fragmentation, which generates the dicarbonyl compound **28** [13,14]. Subsequent β-elimination leads to glycolate **26** (Fig. 12). Thus, diluted aqueous NH_3 converted compound **24** into compound **26**.

Analogous to bleomycin-induced DNA cleavage, the formation of glycolate **26** from peroxide **25** could occur via a Criegee rearrangement (**25**→**29**) and subsequent

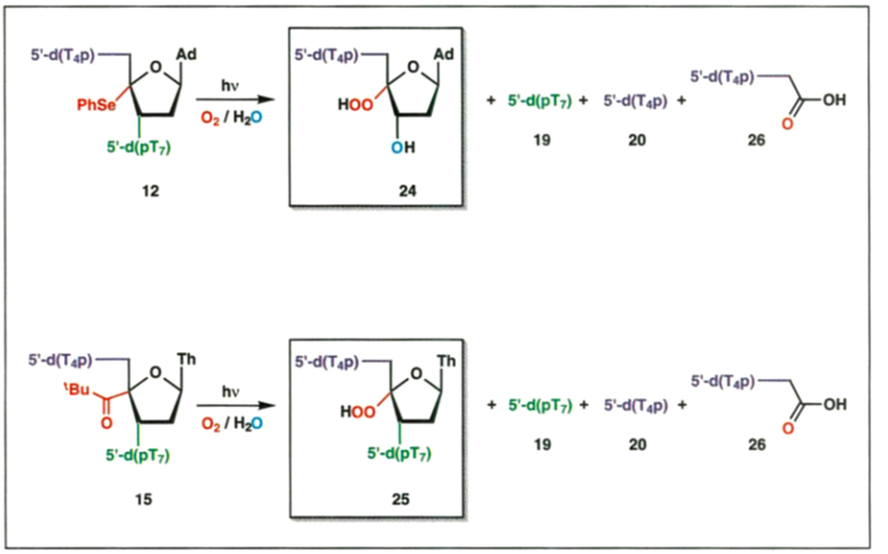

Fig. 8. Products from single-stranded 4'-DNA radicals **14** and **17** (generated by photolysis of compounds **12** and **15**, respectively) in the presence of O_2. Different hydroperoxides (**24** or **25**) are formed from different precursors.

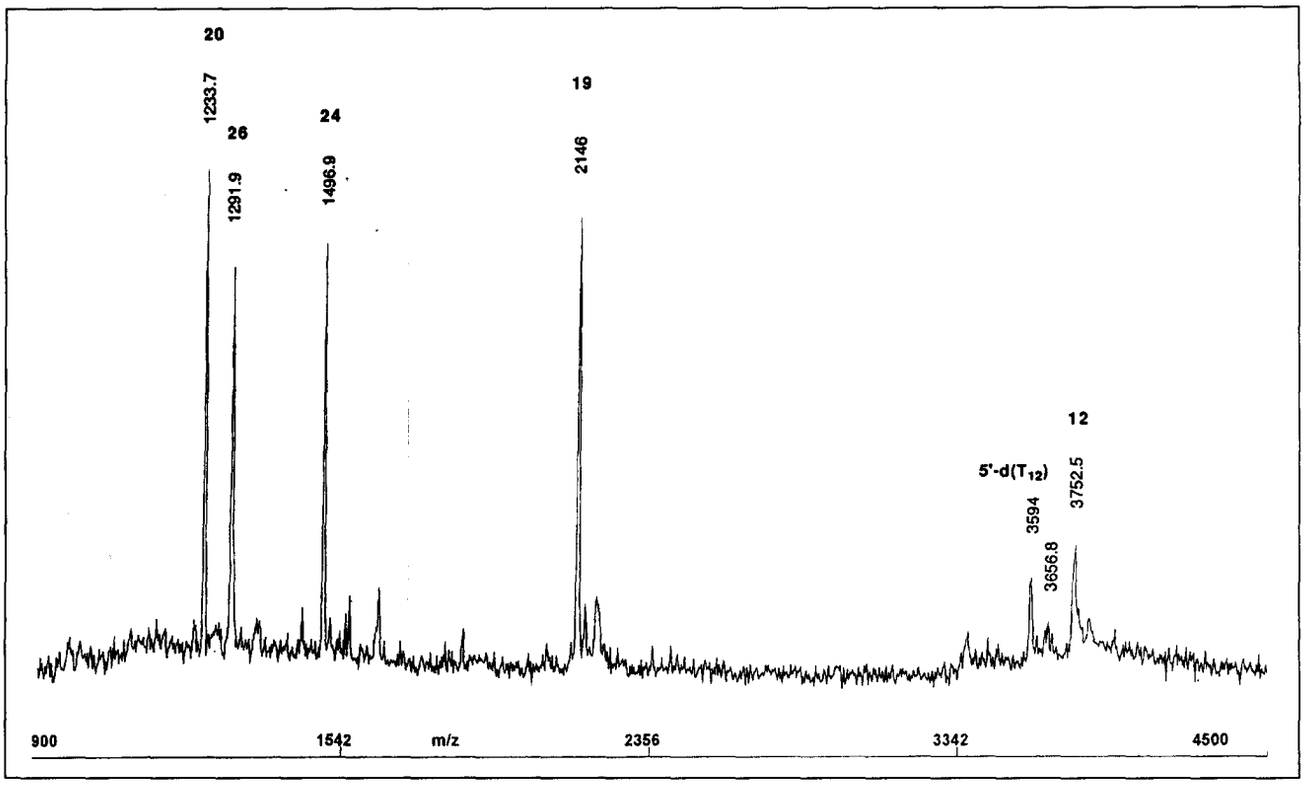

Fig. 9. MALDI-MS of the reaction products of single-stranded 4'-DNA radical **14** (generated by photolysis of compound **12**) in the presence of O_2 (the formulae of the compounds are shown in Figs 8 and 11). Hydroperoxide **24** of the cleaved DNA strand is formed. The peak with *m/z* = 3656.8 is not a signal of the hydroperoxide of the uncleaved strand.

β-elimination [1,4,15]. As expected for such a mechanism, diluted aqueous HCl completely destroyed hydroperoxide **25**. Isotope studies with $^{18}O_2$ and $H_2{}^{18}O$ demonstrated that, starting from precursor **12** as well as from **15**, one of

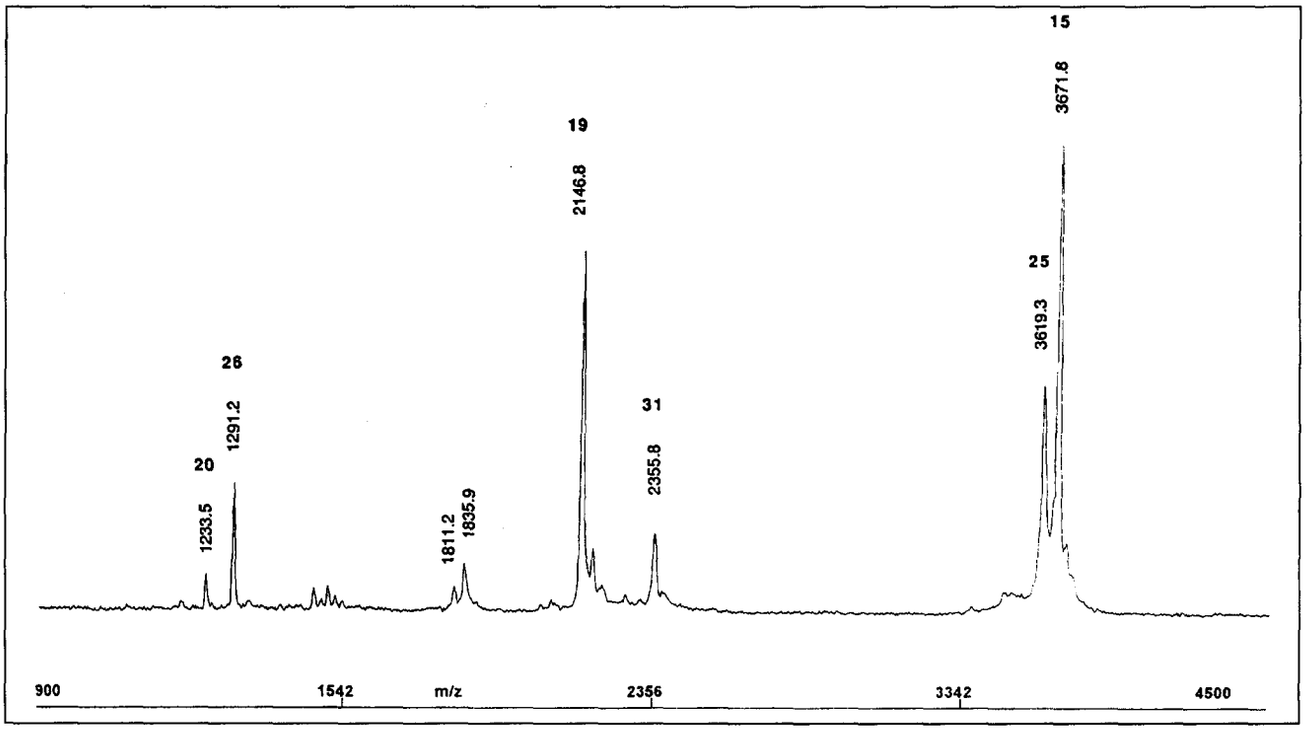

Fig. 10. MALDI-MS of the reaction products of single-stranded 4'-DNA radical **17** (generated by photolysis of compound **15**) in the presence of O_2 (the formulae of the compounds are shown in Figs 8 and 13). The hydroperoxide **25** of the uncleaved strand is formed. The peaks with *m/z* = 1811.2 and 1835.9 correspond to the doubly deprotonated compounds **25** and **15**.

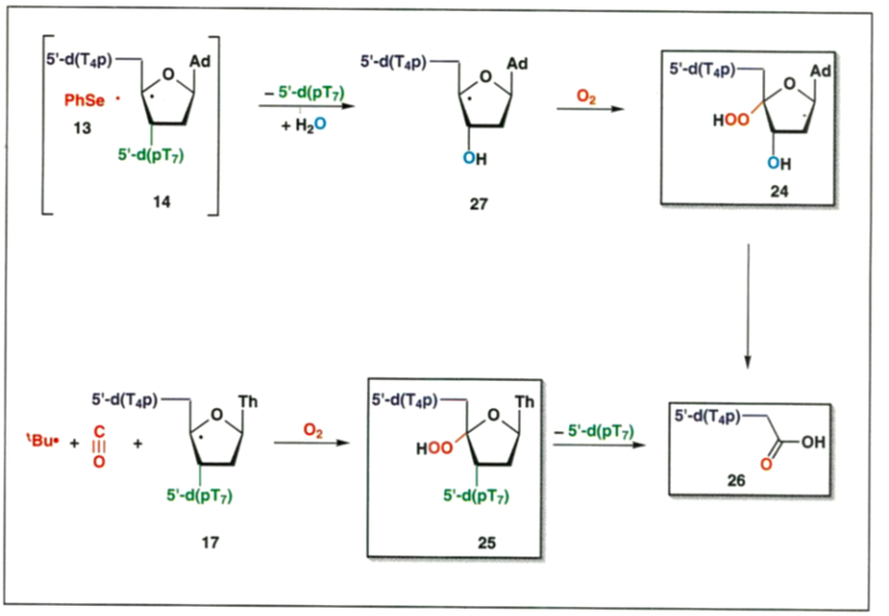

Fig. 11. Formation of hydroperoxides **24** and **25** from single-stranded 4'-DNA radicals **14** and **17** in the presence of O_2. Both hydroperoxides are precursors of glycolate **26**.

the oxygen atoms of glycolate **26** comes from O_2, but the oxygen atom from H_2O is not incorporated into the glycolate. This is in accord with the suggested mechanisms shown in Figure 12.

The MALDI mass spectrum of the oxidation products of the DNA radical, generated from compound **15** as radical precursor, shows the formation of a further product with m/z = 2355.8 (Fig. 10). According to isotope studies, this compound again contains one of the oxygen atoms from O_2. It is conceivable that this product might also be formed from peroxide **25**. Migration of the 5'-alkyl group in the Criegee rearrangement (**25**→**30**) and a subsequent substitution reaction could lead to lactone **31** (Fig. 13). The mass spectra of a reaction series analyzed after different photolysis times showed that the ratios of the oxidized products **26**/**31** and of the oligomers **19**/**20** are roughly independent upon the reaction time, whereas peroxide **25** reaches a maximum and then decreases. It is therefore highly likely that peroxide **25** is an intermediate that yields oxidized products **26** and **31** in a competing reaction.

To our knowledge, these are the first experiments in which the hydroperoxide of the initially generated 4'-DNA radical could be detected directly. Furthermore, these experiments show that hydroperoxides of degraded oligonucleotides can be formed if the cleavage reactions of single-stranded 4'-DNA radicals are fast. Such a DNA strand cleavage, that is not induced by oxygen, can occur in situations where the 4'-DNA radical is shielded from external attack.

Significance

Many antitumor antibiotics, like enediynes or bleomycin, and artificial reagents that generate

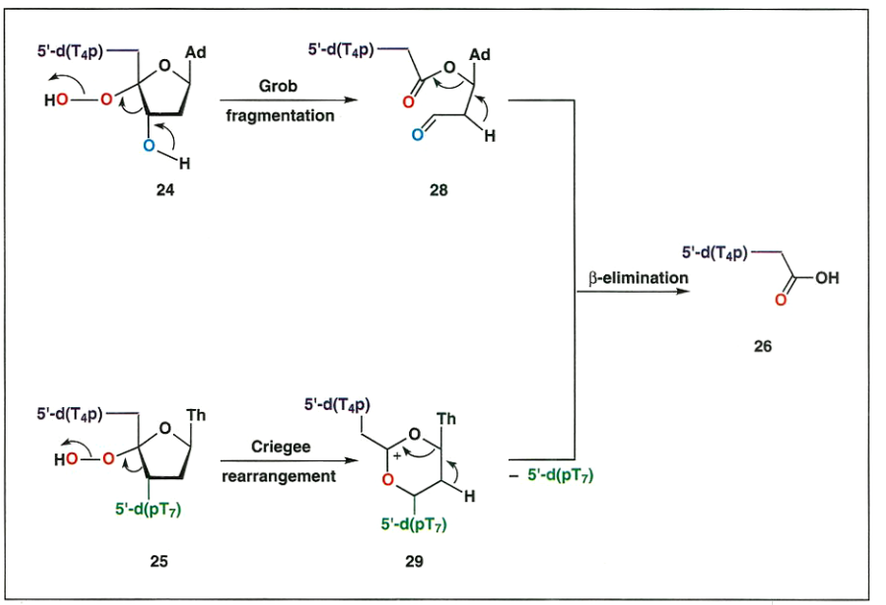

Fig. 12. Proposed mechanism for the formation of glycolate **26** from strand-cleaved hydroperoxide **24** [13] and from hydroperoxide **25** with the intact strand [15].

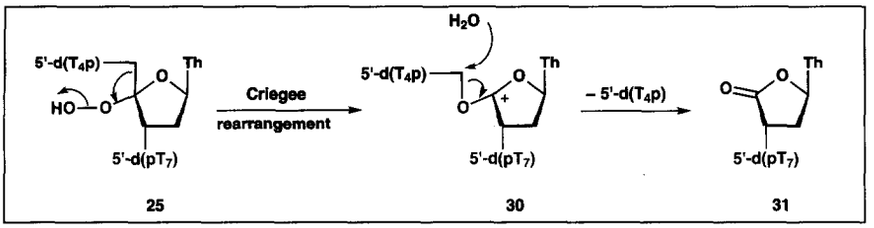

Fig. 13. Possible mechanisms for the formation of lactone **31** from hydroperoxide **25**.

oxygen-centered radicals induce the formation of DNA radicals. Such radicals are cleaved under oxidative conditions [1–4]. We have developed a technique of generating single-stranded 4'-DNA radicals under mild conditions in the absence of external reagents that makes it possible to observe the previously undetected intermediate hydroperoxides and reaction products. We have therefore been able to prove the existence of DNA hydroperoxides and study their decomposition pathways.

We have shown that, under certain conditions, the single-stranded DNA radical cleaves the DNA strand before the trapping reaction by O_2 occurs. This is the case if the DNA radical is generated in a radical pair and is therefore shielded from external attack. Radical-generating reagents that bind tightly to DNA could therefore have a different effect in the DNA cleavage reaction than loose binders.

With this new method of direct generation of 4'-DNA radicals and the information gained on the mechanism of oxidative cleavage of a single strand, it should now be possible to make progress in understanding the radical-induced cleavage of double-stranded DNA.

Materials and methods

Preparation of modified oligonucleotides **12** *and* **15**

The selenated oligonucleotide **12** and the acylated oligonucleotide **15** were prepared from the modified mononucleosides [16,17] by automated synthesis on an ABI 392 DNA synthesizer in a 0.2 μmol scale (20 mol equivalents amidite per cycle, 500 Å CPG support) as previously described [5].

MALDI-MS analysis of educts and products

The experiments were conducted with a Vestec Benchtop II instrument in the negative-ion method. Matrix solution: 2,4,6-trihydroxyacetophenone (0.3 M) or 2,4-dihydroxyacetophenone (0.3 M) in CH_3CN:H_2O:EtOH = 50:45:5. The samples were prepared by mixing 1 μl of the matrix solution and 0.5 μl of a 0.3 M solution of ammonium tartrate in H_2O. After evaporation to dryness the mass spectra were recorded with a laser intensity of 0.2 μJ per pulse (about 20 to 50 pulses were accumulated per recording). The pulse time was 3 ns (λ_{laser} = 337 nm).

Photolysis of compounds **12** *and* **15** *in the presence of glutathione*

A degassed aqueous solution of compound **12** or **15** (100–200 μl, A_{260} = 3–5 OD) containing 0.0 to 2.0 mg of glutathione was irradiated (Osram high pressure lamp, 500 W, 320 nm filter) at 20 °C. Samples were taken after 5, 10, 20, 40 and 90 min and analyzed by MALDI-MS (see Figs 5–7).

Photolysis of compound **12**: MALDI-MS *m/z* 3751.6 (**12**, calc'd [M-H]$^-$: 3751.5); 3672.7, (**22**, calc'd [M-H]–: 3672.5); 3596.9 (**18**, calc'd [M-H]$^-$: 3596.4); 2145.8 (**19**, calc'd [M-H]$^-$: 2147.4); 1466.5 (**21**, calc'd [M-H]$^-$: 1467.0); 1233.5 (**20**, calc'd [M-H]$^-$: 1233.8).

Photolysis of compound **15**: MALDI-MS *m/z* 3671.5 (**15**, calc'd [M-H]$^-$: 3671.5); 3588.6 (5'-d(T_{12}), calc'd [M-H]$^-$: 3587.4); 2145.5 (**19**, calc'd [M-H]$^-$: 2147.4); 1457.3, (5'-d(T_5) calc'd [M-H]$^-$: 1458.0); 1231.9 (**20**, calc'd [M-H]$^-$: 1233.8).

Photolysis of compounds **12** *and* **15** *in the presence of* O_2

An aqueous solution of compound **12** or **15** (100–200 μl; A_{260} = 3–5 OD) was saturated with O_2 (about 1 mM) and irradiated (Osram Hg high pressure lamp, 500 W, 320 nm filter) at 20 °C. Samples were taken every 5 min and analyzed by MALDI-MS (see Figs 9 and 10).

Photolysis of compound **12**: MALDI-MS *m/z* 3752.5 (**12**, calc'd [M-H]$^-$: 3751.5); 3594 (5'-d(T_{12}), calc'd [M-H]$^-$: 3587.4); 2146 (**19**, calc'd [M-H]$^-$: 2147.4); 1496.9 (**24**, calc'd [M-H]$^-$: 1499.0); 1292 (**26**, calc'd [M-H]$^-$: 1291.5); 1233.9 (**20**, calc'd [M-H]$^-$: 1233.8).

Photolysis of compound **15**: MALDI-MS *m/z* 3671.8 (**15**, calc'd [M-H]$^-$: 3671.5); 3619.3 (**25**, calc'd [M-H]$^-$: 3619.6); 2355.8 (**31**, calc'd [M-H]$^-$: 2354.9); 2146.8 (**19**, calc'd [M-H]$^-$: 2147.4); 1291.2 (**26**, calc'd [M-H]$^-$: 1291.5); 1233.5 (**20**, calc'd [M-H]$^-$: 1233.8).

Experiments with $^{18}O_2$ and/or $H_2{}^{18}O$ were performed under the same conditions. In a series of experiments, it was demonstrated that with $^{18}O_2$ in H_2O the mean value of the mass numbers was increased by two units for the peaks of compounds **26** and **31**, and by four units for compounds **24** and **25**. In $H_2{}^{18}O$ only the peak of compound **24** was increased by two mass numbers. Experiments with $^{18}O_2$ in $H_2{}^{18}O$ increased the mean values of the peaks of compounds **26** and **31** by two mass units, compound **25** by four units and compound **24** by six units.

Treatment of hydroperoxide **24** *with* NH_3

An aqueous solution of compound **12** (100 μl; A_{260} = 5 OD) was saturated with O_2 (about 1 mM) and irradiated (Osram Hg high pressure lamp, 500 W, 320 nm filter) at 15 °C. After 40 min, a sample (1 μl) was analyzed by MALDI-MS and showed the formation of the hydroperoxide **24** as one of the reaction products. The remaining solution was treated with an aqueous solution of NH_3 (28 %, 100 μl) for one hour. After freeze-drying, the residue was dissolved in H_2O and analyzed by MALDI-MS. The hydroperoxide **24** could no longer be detected and the peak of the glycolate **26** had increased. In

test experiments without NH_3 we demonstrated that the freeze-drying process lowers the amount of hydroperoxide **24** only marginally.

*Treatment of hydroperoxide **25** with HCl*

An aqueous solution of compound **15** (150 μL; A_{260} = 5 OD) was saturated with O_2 (about 1 mM) and irradiated (Osram Hg high pressure lamp, 500 W, 320 nm filter) at 15 °C. Two samples each were taken after 5, 10, 15, 20, 25 and 30 min. The first sample (1 μl) of the irradiated solution was analyzed by MALDI-MS and showed the formation of the hydroperoxide **25** as one of the reaction products. The second sample (1 μl) was treated with 1 μl of HCl (1 M) for 5 min. Then 1 μL of a 2,4-dihydroxyacetophenone solution (0.3 M, CH_3CN:H_2O:EtOH = 50:45:5) was added, and the solution evaporated to dryness. MALDI-MS revealed that the hydroperoxide **25** was destroyed by this treatment with acid.

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