ORGANIC LETTERS

2004
Vol. 6, No. 14
2329−2332

Generation of a C-3′-Thymidinyl Radical in Single-Stranded Oligonucleotides under Anaerobic Conditions

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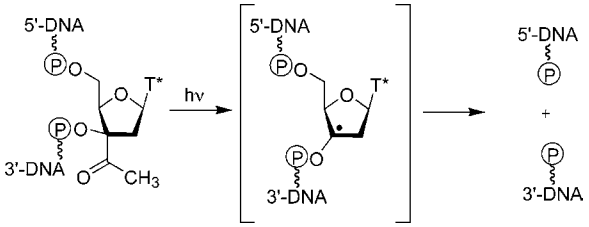
Received April 9, 2004

ABSTRACT



A C-3′-thymidinyl radical has been photochemically generated site-specifically in DNA oligonucleotides. A nucleoside H-phosphonate bearing a C-3′ acetyl group was incorporated into DNA oligomers using a hand-coupling technique. When nucleotides containing the modified monomer were photolyzed (≥320 nm) in the presence of a hydrogen atom donor, reduction products were detected by RP-HPLC and MALDI-ToF MS analysis.

Understanding the consequences of oxidative damage to cellular components is essential in the determination of disease etiology. Of the different forms of possible damage, oxidative assault on DNA has been determined to play a significant role in the development of disease.[1] Due to this fact, a great deal of effort has been devoted to obtaining a better understanding of this process through the introduction of techniques for the specific generation of reactive intermediates in DNA. These methods facilitate the analysis of their subsequent mechanisms of decomposition as well as their role in further damage.[2] Several carbohydrate-based DNA radicals have been studied in great detail under both aerobic and anaerobic conditions, with the C-1′[3] and C-4′[4] species being studied most extensively. All five possible sugar radicals have been found to lead to cleavage of the DNA strand either directly or after base treatment.[5]

This report details our successful independent generation and study of one of the deoxyribose radicals that has received less attention, the C-3′-DNA radical (**1**). A significant body of work involving this reactive species, which is generated by the abstraction of a C-3′-hydrogen from a deoxyribonucleotide, is related to the investigation of photonucleases designed by Barton and co-workers.[6] Upon activation with light, these Rh−phenanthrenequinone diimine complexes abstract the most available hydrogen atom in the major groove, the 3′-hydrogen, causing strand scission. The mech-

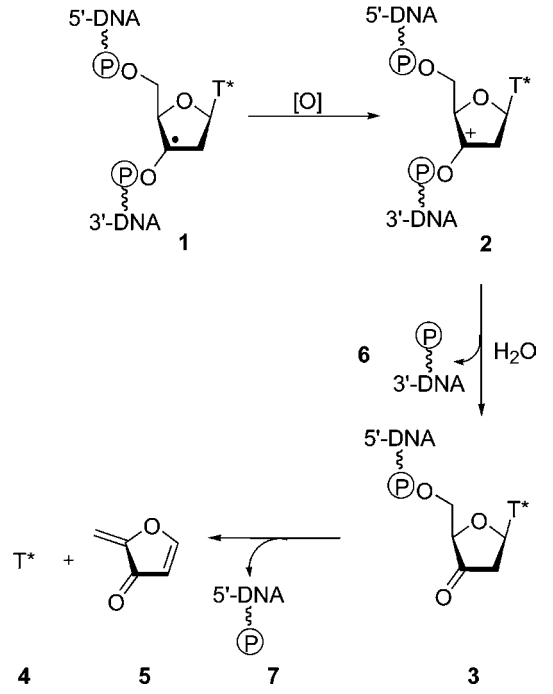
(1) Marnett, L. J. *Toxicology* **2002**, *181*−*182*, 219−222. Marnett, L. J.; Riggins, J. N.; West, J. D. *J. Clin. Invest.* **2003**, *111*, 583−593.
(2) Greenberg, M. M. *Chem. Res. Toxicol.* **1998**, *11*, 1235−1248.

(3) (a) Sigman, D. S.; Graham, D. G.; D'Aurora, V.; Sterm, A. M. *J. Biol. Chem.* **1979**, *254*, 12269−12272. (b) Goyne, T. E.; Sigman, D. S. *J. Am. Chem. Soc.* **1987**, *109*, 2846−2848. (c) Meijler, M. M.; Zelenko, O.; Sigman, D. S. *J. Am. Chem. Soc.* **1997**, *119*, 1135−1136. (d) Zelenko, O.; Gallagher, J.; Sigman, D. S. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 2776−2778. (e) Tronche, C.; Goodman, B. K.; Greenberg, M. M. *Chem. Biol.* **1998**, *5*, 263−271. (f) Tallman, K. A.; Tronche, C.; Yoo, D. J.; Greenberg, M. M. *J. Am. Chem. Soc.* **1998**, *120*, 4903−4909. (g) Chen, T.; Greenberg, M. M. *J. Am. Chem. Soc.* **1998**, *120*, 3815−3816. (h) Greenberg, M. M.; Yoo, D. J.; Goodman, B. K. *Nucleosides Nucleotides* **1997**, *16*, 33−40. (i) Goodman, B. K.; Greenberg, M. M. *J. Org. Chem.* **1996**, *61*, 2−3. (j) Hwang, J.-T.; Tallman, K. A.; Greenberg, M. M. *Nucleic Acids Res.* **1999**, *27*, 3805−3810. (k) Hashimoto, M.; Greenberg, M. M.; Kow, Y. W.; Hwang, J.-T.; Cunningham, R. P. *J. Am. Chem. Soc.* **2001**, *123*. (l) Chatgilialoglu, C.; Gimisis, T. *Chem. Commun.* **1998**, 1249−1250. (m) Emanuel, C. J.; Newcomb, M.; Ferreri, C.; Chatgilialoglu, C. *J. Am. Chem. Soc.* **1999**, *121*, 2927−2928. (n) Chatgilialoglu, C.; Ferreri, C.; Bazzanini, R.; Guerra, M.; Choi, S.-Y.; Emanuel, C. J.; Horner, J. H.; Newcomb, M. *J. Am. Chem. Soc.* **2000**, *122*, 9525−9533.

10.1021/ol0493453 CCC: $27.50
Published on Web 06/05/2004

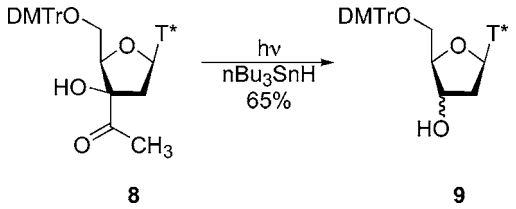
anism of cleavage in the presence of these metal complexes is shown in Scheme 1.[6]

Scheme 1



It has been proven that a highly efficient means of site-selectively generating reactive intermediates in biological molecules is through photolysis of synthetically modified nucleosides that are incorporated into oligomers.[2] Giese et al.[7] demonstrated that a C-3′-thymidinyl radical could be successfully generated from a radical precursor that contains an acetyl moiety at the C-3′-position (**8**) (Scheme 2). Even though this monomer possesses the unnatural xylo configuration, it was shown that upon reduction, a 1:1 mixture of xylo:ribo products is formed. This indicates that the C-3′-radical is equally accessible from both sides of the molecule and the conformation of the starting nucleoside should have little effect on the outcome of our studies. Here, we report the successful use of **8** for the incorporation of an acetyl-modified thymidine into single-stranded DNA and its

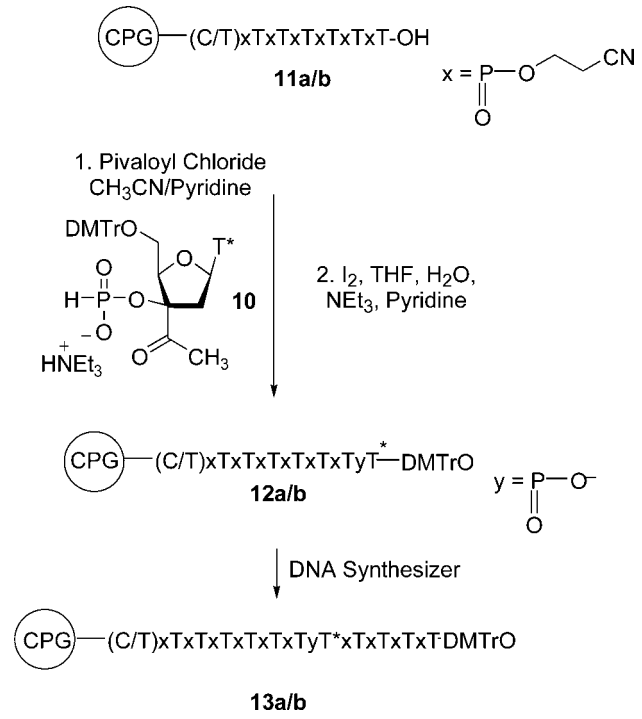
Scheme 2



subsequent use in the generation and investigation of a C-3′-DNA radical.

DNA oligomers, **O1** (5′-TTTTT*TTTTTTT-3′) and **O2** (5′-TTTTT*TTTTTTC-3′), were synthesized for photochemical studies. The modified nucleoside was placed in position five of each of the oligomers to ease analysis in the case of strand scission (Scheme 3). With this design, fragments

Scheme 3



resulting from degradation of the sugar containing the initially generated radical would be, in the case of **O1** and **O2**, TTTT-3′-phosphate and TTTTTTT- for **O1** or TTTTTTC-5′-phosphate for **O2**. These possess significantly different molecular masses, making them easily distinguishable using MALDI-ToF MS. Polyd(T) strands were chosen as a starting point for these studies due to the efficiency of their synthesis and high stability.

The standard methodology utilized in the automated synthesis of oligonucleotides uses phosphoramidite monomers. The synthesis of such monomers containing substituents at the C-3′-position of the sugar is, however, not trivial. This is due to steric limitations imposed by both the presence

(4) (a) Giese, B.; Burger, J.; Kang, T. W.; Kesselheim, C.; Wittmer, T. *J. Am. Chem. Soc.* **1992**, *114*, 4. (b) Giese, B.; Beyrich-Graf, X.; Burger, J.; Kesselheim, C.; Senn, M.; Schaefer, T. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1742−1743. (c) Crich, D.; Mo, X.-S. *Tetrahedron Lett.* **1997**, *38*, 8169−8172. (d) Giese, B.; Beyrich-Graf, X.; Erdmann, P.; Schwitter, U. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 1861−1863. (e) Giese, B.; Dussy, A.; Meggers, E.; Petretta, M. *J. Am. Chem. Soc.* **1997**, *119*, 11130−11131. (f) Giese, B.; Beyrich-Graf, X.; Erdmann, P.; Petretta, M.; Schwitter, U. *Chem. Biol.* **1995**, *2*, 367−375. (g) Giese, B.; Beyrich-Graf, X.; Erdmann, P.; Giraud, L.; Imwinkelried, P.; Muller, S. N.; Schwitter, U. *J. Am. Chem. Soc.* **1995**, *117*, 6146−6147. (h) Dussy, A.; Meggers, E.; Giese, B. *J. Am. Chem. Soc.* **1998**, *120*, 7399−7403. (i) Crich, D.; Yao, Q. *Tetrahedron* **1998**, *54*, 305−318. (j) Schiemann, O.; Feresin, E.; Carl, T.; Giese, B. *Chemphyschem* **2004**, *5*, 270−274.

(5) Pogozelski, W. K.; Tullius, T. D. *Chem. Rev.* **1998**, *98*, 1089−1107.

(6) Sitlani, A.; Long, E. C.; Pyle, A. M.; Barton, J. K. *J. Am. Chem. Soc.* **1992**, *114*, 2303−2312.

(7) Körner, S.; Bryant-Friedrich, A.; Giese, B. *J. Org. Chem.* **1999**, *64*, 1559−1564.

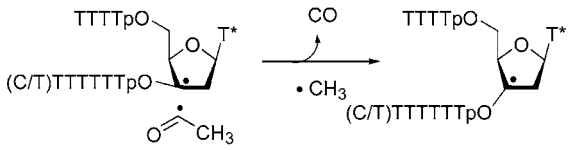
of the C-3′-modification and the size of the phosphoramidite moiety commonly used in this method of automated DNA synthesis. Also, electronic factors such as those created by the incorporation of the site of phosphitylation into a α-hydroxyl keto moiety disfavor this method. For this reason we chose to use the H-phosphonate method for the incorporation of C-3′-acetylthymidine.[8] In addition, due to the decreased reactivity of the 3′-hydroxyl, the use of the more reactive phosphitylating reagent derived from phosphorus trichloride seemed warranted. These conditions produced H-phosphonate **10** as its triethylammonium salt in 78% yield. Even with this very reactive reagent, extended reaction times and elevated temperatures as compared to the conditions used for the phosphitylation of unmodified nucleosides were required (See Supporting Information).

To develop conditions that would be used in the future for the incorporation of C-3′-acetylthymidine H-phosphonate **10** into DNA oligomers using automated synthesis, we examined a manual technique for our initial experiments (see Supporting Information). Such an approach gave us the flexibility required to easily vary reagent concentrations and coupling times. A polymer-bound oligonucleotide (**11**) was constructed on a DNA synthesizer using the phosphoramidite method (Scheme 3). The synthesis of these unmodified oligonucleotides proceeded with coupling efficiencies of 99%. Compound **10** was then incorporated using a manual syringe technique employing reagents and conditions described for the H-phosphonate method.[8] The modified H-phosphonate was dissolved in an appropriate amount of 1:1 acetonitrile/pyridine and placed in a syringe. In a separate syringe was placed the activator needed for nucleoside coupling, pivaloyl chloride, in the same solvent mixture. These syringes were attached to each end of the CPG column containing oligomer **11a** or **11b**, making it possible to simultaneously expose these coupling partners to both H-phosphonate and activator in varying concentrations and reaction times. CPG-bound oligomers **12a** and **12b** were obtained after oxidation of the H-phosphonate diesters to their corresponding phosphates through simultaneously exposing the polymer-bound oligomers to a 0.2 M solution of iodine in THF and a 1:1:8 mixture of water/triethylamine/THF. When further DNA synthesis is attempted without oxidation of the H-phosphonate, hydrolysis of the phosphodiester bond and extrusion of the newly introduced modified nucleoside is observed. Incorporation of the modified nucleoside occurs with a coupling efficiency of ~90%. The remainder of the oligomer was added on the DNA synthesizer using the phosphoramidite method with retention of the final dimethoxytrityl protecting group. Cleavage of the oligonucleotide from the solid support and deprotection followed standard procedures. Purification was performed using RP-HPLC and the products identified by MALDI-ToF MS (see Supporting Information).

Upon photolysis of single-stranded oligomers **O1**/**O2** in aqueous solution at ≥320 nm (see Supporting Information), the C-3′-acetyl group undergoes a Norrish type I fragmentation that generates the acetyl radical along with DNA radical **14**/**15** (Scheme 4). To evaluate the performance of the C-3′-acetyl group as a radical precursor, **O2** (2 nmol) was irradiated under anaerobic conditions in the presence of a large excess (1000 equiv) of glutathione (GSH). About 50% of the modified nucleoside is consumed in 30 min. Direct analysis by MALDI-ToF MS revealed the presence of the expected isomeric reduction products **O3** and **O4** (calcd 3571.4, obsd 3570.6 amu) (Scheme 5). Irradiation of the same oligomer (2 nmol) under similar conditions, however, with only 500 equiv of GSH delivered similar results. Strand cleavage products were also identified in both cases. Approximately 10% of the radical formed was converted to 3′- and 5′-phosphate. These products may be the result of an alternate mechanism that involves the C-3′-radical or another reactive intermediate.

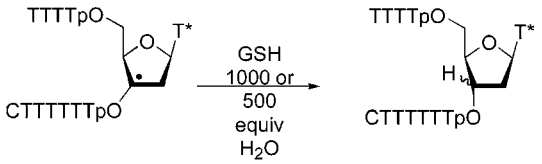
Having ascertained that these modified nucleotides produce the radical of interest, we then irradiated **O1** and **O2** under anaerobic conditions in the absence of an H-donor. Direct analysis of the reaction mixture after photolysis, using MALDI-ToF MS revealed the generation of several fragments. The major fragments were identified as 3′-phosphate cleavage product **16** and 5′-phosphate cleavage products **17** and **18** (~40% yield) (Scheme 6). According to the mecha-

Scheme 4



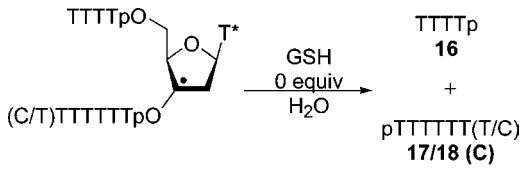
O1/O2 (C) **14/15 (C)**

Scheme 5



15 **O3/O4**

Scheme 6



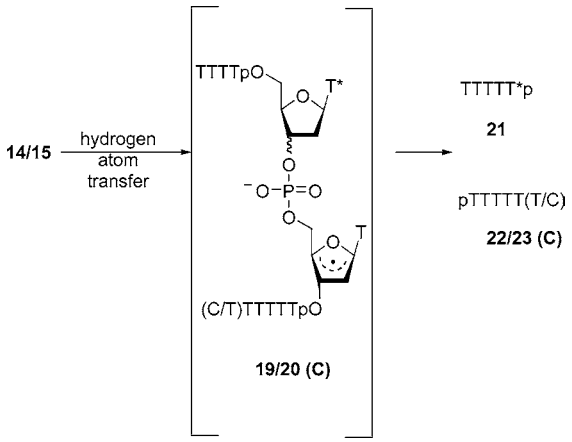
14/15

(8) *Current Protocols in Nucleic Acid Chemistry: Synthetic Strategies and Parameters Involved in the Synthesis of Oligodeoxyribo- and Oligoribonucleotides According to the H-Phosphonate Method*; Stroemberg, R., Stawinski, J., Eds.; John Wiley & Sons: New York, 2002; Vol. 1.

nism shown in Scheme 1, products **16** and **17**/**18** are to be expected from the decomposition of the C-3′-DNA radical. A molecular ion peak was also observed, which corresponds to ketone-containing fragment **3** shown in Scheme 1. This fragment is the result of the oxidation and subsequent hydrolysis of the photochemically generated radical.[6] The formation of this fragment is a prerequisite for the formation of 3′-phosphate **16**. It is not completely clear at this time what the oxidant source is in this process. This question is currently under investigation.

The mechanism shown in Scheme 1 does not, however, explain the formation of 3′-phosphate **21** or 5′-phosphate **22**/**23** (Scheme 7). These products, observed in the photolysate of both **O1** and **O2** not withstanding H-donor concentration, are believed to arise from hydrogen atom transfer to the originally generated C-3′-radical from its 3′-adjacent nucleotide. The spontaneous formation of DNA strand scission products would indicate that the radical is carbohydrate in nature. Since this appears to be a minor reaction pathway for this thymidinyl radical, very careful analysis of minor reaction products will be carried out to determine the nature of this radical.

Scheme 7



In summary, we have shown that C-3′-radicals can be generated site-specifically in 2′-deoxyoligonucleotides using a C-3′-acetyl-modified thymidine H-phosphonate. The use of this nucleotide allows the incorporation of a radical precursor at a predetermined location within the oligomer using the H-phosphonate method. Our initial investigations indicate that photochemical generation of this radical leads to spontaneous strand scission. In addition, this reactive intermediate participates in a hydrogen atom transfer generating a new radical that also leads to spontaneous strand scission. The mechanistic aspects of this radical transfer process are currently under investigation.

Acknowledgment. I thank B. Giese and S. Körner for many helpful discussions at the beginning of this project and the National Science Foundation (CAREER: CHE-0239525), Oakland University, and the Oakland University Biomedical Research Center for funding.

Supporting Information Available: Experimental procedures, HPLC traces, and MALDI-ToF spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

OL0493453