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Communications to the Editor

Hydrogen Peroxide Oxidation of Phosphite Triesters in Oligonucleotide Syntheses

Raymond J. Cvetovich*

Department of Process Research, Merck Research Laboratories, Rahway, New Jersey 07065, U.S.A

Abstract:

The oxidation of P(III) phosphite triesters to stable P(V) phosphates in oligonucleotide syntheses using hydrogen peroxide was demonstrated in neutral aqueous acetonitrile as a cheap, green alternative to the widely used iodine pyridine procedure. This oxidation was utilized on short oligomers of two nucleosides.

Introduction

Short interfering ribose nucleic acids (siRNA) have the ability to specifically suppress cellular gene expression and hold great promise as target validation for small molecules as well as RNAi-based therapy for disease-causing genes.[1] The most commonly used methodology for the synthesis of both DNA and RNA employs the phosphoramidite techniques introduced by Beaucage.[2] As interest in siRNA has grown, the importance of developing cheap, robust and, where possible, green processes used in the production of these oligonucleotides has increased. Iodine/pyridine/water (0.05 M/90 vol %/10 vol %) is the standard protocol in oligonucleotide syntheses for the oxidation of the newly created P(III) triester created by coupling with phosphoramidites.[3] Alternative oxidants have been examined and reported in the literature. Fourrey and Varenne[4] described the use of iodobenzene diacetate and tetrabutylammonium periodate to oxidize phosphite triesters. Hayakawa, Uchiyama and Noyori[5] examined the use of TMSOOTMS, H_2O_2, *tert*-butyl hydroperoxide, cumene peroxide, di-*tert*-butyl peroxide, trimethylamine *N*-oxide, *N*-methylmorpholine-*N*-oxide, pyridine *N*-oxide, and dimethylsulfoxide. They also described the use of TMSOTf- and Nafion-TMS-assisted oxidations. All of these oxidations were examined under anhydrous conditions on coupled 2-mers in solution phase, and yields of up to 95% were realized under a variety of time and temperature conditions. The need for anhydrous conditions is suspect, in that iodine is typically used as a 10% aqueous pyridine solution. Success in using this reagent lies in the kinetics, oxidation being faster than hydrolysis of the P(III) esters. Noyori[3] reported that a 94% yield was achieved in 5 min at 0 °C with anhydrous hydrogen peroxide oxidation in ether. This success in using hydrogen peroxide appears to have been ignored due the difficulty in producing anhydrous hydrogen peroxide in ether, let alone the danger of producing such a reagent by the methods employed. Among other oxidants having received attention in the literature, Caruthers[6] has demonstrated the use of peroxyanions to both oxidize phosphites and remove 5′-*O*-carbonate protecting groups; Sanghvi[7] has reported the use of NBS−DMSO in acetonitrile under nonbasic and nonaqueous conditions; Hayakawa[8] discussed the advantages of ethyl(methyl)dioxirane, and Manoharan[9] has used (1*S*)-(+)-10-camphorsulfonyloxaziridine. Most recently, Sekine has reported the use of 1,1-dihydroperoxycyclodecane as a crystalline nonhygroscopic oxidizer.[10]

Results and Discussion

To test and compare the usefulness of hydrogen peroxide in the oxidation of P(III)-phosphite triesters in oligonucleotide synthesis, a series of single amidite additions to 2′-*O*-methyl uridine attached to controlled pore glass (CPG) was studied (see Figure 1). The addition of a single amidite has the advantage of ease of analysis and quantitation of the products formed in each reaction. As the control, iodine (0.05 M in 10% water in pyridine) was used to oxidize the P(III) triester formed, and the yield of product, as measured by HPLC, was compared to the yield from oxidation with solutions of urea peroxide (∼0.25 M in 1.5% water in acetonitrile).[11] Performing the synthesis on an Akta OP-10 (GE Healthcare) on 100 μmol scale at 20−25 °C, oxidation was accomplished using a one-column volume of either iodine (method A) or urea peroxide solutions (method

* Author for correspondence. E-mail: rcvetovich@gmail.com.

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(11) 1.5 vol% water is necessary to dissolve the urea peroxide to achieve a concentration of 0.25M. Anhydrous acetonitrile solutions of urea peroxide have also been successfully used as saturated solutions of ∼0.08M concentration.

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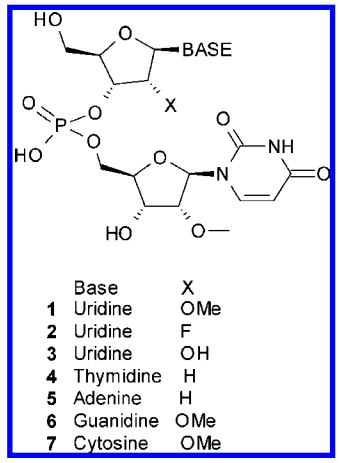

Figure 1. **2-Mers produced with iodine or hydrogen peroxide oxidation.**

B), added at the same rates (1 mL/min, 6.3 mL of solution to a 6.3 mL volume synthesis cartridge).

The 2-mers produced (**1**−**7**) were detritylated, cleaved from the CPG with 40% methylamine in water[12] at 35 °C for 45 min, filtered to remove the spent glass, and then assayed by HPLC. After calculating the area counts per μmol for each reaction (similar to the ODs calculated for the syntheses of longer oligonucleotides), the production of product was compared for the oxidation procedure used. For the initial addition of 2′-*O*-methyl uridine phosphoramidite the amount of [omeU]-PO_2-[omeU] 2-mer **1** was found to be equivalent (8200 vs 8040 area counts per μmol, entries 1, 2 in Table 1). The influence of other phosphoramidites (2′-fluorouridine, deoxythymidine, and 2′-OTBDMS ribouridine) was also examined using the same comparison analysis. Each reaction was performed in the manner described above and analyzed by HPLC for the amount of product formed, the results of which are tabulated in Table 1.

In comparing oxidation procedure A and B, the iodine oxidation (method A) produced more product than urea peroxide (method B) in varying amounts (see entries 1 vs 2, 5 vs 6, 9 vs 10, 13 vs 14). The greatest difference was observed with 2′-OTBDMS ribouridine (entries 5, 6), and this difference could be attributed to the steric environment and the difference in the rate of peroxide vs iodine oxidation. Because the typical iodine oxidation method used in oligonucleotides syntheses is performed with 10 vol % water, solutions of hydrogen peroxide with higher water content were prepared by adding 50% H_2O_2 to acetonitrile to prepare 0.25 M peroxide solutions containing 8.5 vol % water (method C). The above phosphoramidites were again coupled to 2′-OMe-uridine on CPG and oxidized with the new solution using the same volume and rate of pumping as before and compared to the previous reactions. As can be seen in entries 3, 7, 11, and 15, using method C, the amount of product produced in several cases was less than that with either methods A or B.

(12) In the case of adenine, guanidine and cytosine, deprotection also occurred under these conditions. For O-TBDMS deprotection of **3** desilylation preceded assays. See Vargeese, Chandra; Shaffer, Christopher; Bowman, Keith. Rapid one-pot method for deprotection of ribonucleotide-containing oligonucleotides using aqueous methylamine and followed by triethylammonium trihydrofluoride. PCT Int. Appl. (2004), 74 pp.

Table 1. **Phosphoramidite addition to 2′-OMe uridine on CPG**

entry	phosphoramidite	oxidation method	2-mer product	area counts per μmol	LCAP
1	2′-OMe-uridine	A	**1**	8200	92.9
2	2′-OMe-uridine	B	**1**	8045	89.7
3	2′-OMe-uridine	C	**1**	8170	86.5
4	2′-OMe-uridine	D	**1**	8020	87.0
5	2′-fluorouridine	A	**2**	8970	94.0
6	2′-fluorouridine	B	**2**	8760	90.4
7	2′-fluorouridine	C	**2**	8335	82.8
8	2′-fluorouridine	D	**2**	9050	85.9
9	2′-OTBDMS-uridine	A	**3**	8555	91.4
10	2′-OTBDMS-uridine	B	**3**	8020	84.5
11	2′-OTBDMS-uridine	C	**3**	7580	90.1
12	2′-OTBDMS-uridine	D	**3**	8835	82.2
13	deoxy-thymidine	A	**4**	8665	92.2
14	deoxy-thymidine	B	**4**	8355	86.9
15	deoxy-thymidine	C	**4**	8900	88.0
16	deoxy-thymidine	D	**4**	9245	79.2
17	deoxy-adenine	A	**5**	11460	89.5
18	deoxy-adenine	D	**5**	11265	83.5
19	2′-OMe-guanosine	A	**6**	9984	92.5
20	2′-OMe-guanosine	D	**6**	9860	92.0
21	2′-OMe-cytosine	A	**7**	6786	85.6
22	2′-OMe-cytosine	D	**7**	6926	87.1

Method A: one column volume of iodine (0.05M in 10% water in pyridine); method B: one column volume of urea peroxide (0.25 M in 1.5% water in acetonitrile); method C: one column volume of hydrogen peroxide (0.25 M in 8.5% water in acetonitrile); method D: hydrogen peroxide (0.25 M in 8.5% water in acetonitrile) one column volume, then 10 min recycle.

The amount of water in the mixture could slow down the rate of oxidation or increase the rate of hydrolysis relative to oxidation, and thus a fourth method was applied to the oxidation reaction. A recycling procedure for the oxidation was incorporated to increase the exposure of the P(III)-coupled intermediate without having to increase the volumes of oxidant used in the oxidation step. After adding the same one column of oxidation solution as in method C, the solution was recycled through the synthesis cartridge for 10 min (method D). Following final detritylation and cleavage from the CPG, the amounts of product were again measured by HPLC, and each reaction was shown to produce as much as or more product than the alternative methods (see entries 4, 8, 12, 16). The most dramatic increase came with rU phosphoramidite coupling (entry 12) which produced more product than the iodine oxidation method. This clearly established that while the oxidation rate using hydrogen peroxide was slower with entry 12, the rate of hydrolysis was not the issue.

In all the reactions presented, no new impurities were observed in the comparison of pyridine/iodine to neutral hydrogen peroxide oxidation. The lower LCAP of the peroxide reaction are due to larger amounts of 3′-phosphate byproduct of the amidites utilized. The CPG reagent containing the initial nucleoside has reactive sites that add the activated amidite. It is believed that more of the 3′-phosphate byproduct is observed during the neutral peroxide oxidation because the P(III) linkage between the amidite and CPG is oxidized in preference to hydrolysis, whereas in the pyridine iodine oxidation, the basic aqueous medium leads to faster hydrolysis compared to oxidation. This was demonstrated when ‘native’ CPG (CPG containing no nucleoside) was treated with dT-amidite, oxidized with either iodine or peroxide, and then cleaved from the CPG. Both oxidation protocols produced 3′-dT-phosphates of the amidite utilized, but greater amounts were produced with peroxide

(typically 5% vs 25% yields for I_2 vs H_2O_2). Capping of reactive sites on the surface of CPG-based oligonucleotides, typically performed by extensive acetylation, proved to be ineffective. Effective capping was realized when commercial CPG-2′-OMe-uridine was initially treated with isopropyl cyanoethyl diisopropylaminoamidite under coupling conditions and then oxidized with hydrogen peroxide prior to coupling. Under these conditions <1 LCAP 3′-PO_3-thymidine was observed for either iodine or peroxide oxidation after coupling with dT-amidite. When 'native' CPG was treated in the same manner, little or no 3′-PO_3-thymidine was found. The conclusion is that commercial CPG-based nucleoside reagents require better capping conditions prior to utilization for oligonucleotide syntheses.

Conclusion

The use of neutral hydrogen peroxide in acetonitrile[13] to oxidize the P(III) triester intermediates in oligonucleotide syntheses and its potential to replace iodine oxidation has been demonstrated on 100 μmol on 2-mers syntheses using a variety of amidite nucleosides. The study of longer strands and alternative solvents remains, but this procedure holds promise as a cheaper and cleaner alternative to traditional pyridine/iodine oxidations on all scales.

(13) Thermal characterization of potential hazards in using 1) urea peroxide (solid, exotherm of 299 cal/g with initiation at ~91°C, negative drop weight test at 300 kg.cm); 2) urea peroxide in 1.0% aqueous acetonitrile (an exotherm of 41 cal/g at 227 °C for 0.1 M urea peroxide in 1% aqueous acetonitrile); or 3) hydrogen peroxide in 8.5% aqueous acetonitrile (44.6 cal/g at 230 °C) were all well outside the 20−25 ° operational conditions employed in these experiments.

Experimental Section

^{1}H, ^{13}C, and ^{31}P NMRs were obtained in D_2O on a Bruker 400 MHz instrument in D_2O using referencing to external standards (dioxane and H_3PO_4). The solid-phase synthesis was conducted on a GE Akta Oligoprocess-10 synthesizer. Cleavage from the solid support controlled pore glass and deprotection was carried out in 40% aqueous methylamine. In the case of RNA containing 3′-OTBDMS, the oligomer was cleaved and deprotected with 40% methylamine, filtered, and washed with DMSO; then desilylation was accomplished by pH adjustment with malonic acid (4.8 M in water) to pH 8.5 followed by the addition of potassium fluoride and heating to 60 °C. Purification was carried out on YMC Gel ODS-A resin (loading in 2% NaCl, eluting with water, and then acetonitrile in water from 0.5 to 10%).

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Supporting Information Available

Copies of NMR data spectra (^{1}H, ^{13}C, ^{31}P) for compounds **1**−**7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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