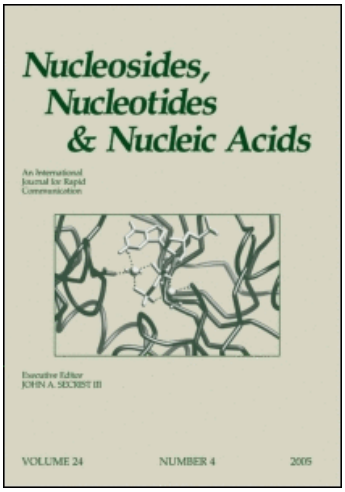

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Synthesis of Antisense Oligonucleotides with Minimum Depurination

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ABSTRACT

The removal of 4,4′-dimethoxytrityl (DMTr) groups from oligonucleotides at low pH and the acid lability of the glycosidic linkage of purine nucleotides constitute an inherent conflict in preparative oligonucleotide chemistry. The use of a mildly acidic NaOAc buffer (10 mM, pH 3.0–3.2) allows adjustment of the pH in a range where the progress of the DMTr removal reaction can be monitored conveniently by HPLC and the optimum reaction time can be calculated. As a result, oligonucleotides with minimum depurination are obtained.

Key Words: Antisense oligonucleotide; DMTr removal; Depurination.

Oligonucleotides (PO-oligos) and their analogs are finding widespread utility in diagnostics, molecular biology, and as therapeutic agents. DNA and RNA analogs, especially phosphorothioate oligonucleotides (PS-oligos) in which one nonbridging oxygen of the natural internucleotide phosphate diester group is replaced by sulfur, are emerging as broadly useful drugs operating through antisense mechanisms of action.[1] PO- and PS-oligos are conveniently prepared by solid phase synthesis using commercially available 5′-*O*-(4,4′-dimethoxytrityl) (DMTr) protected nucleoside 2′-*O*-phosphoramidites as the starting materials.[2] During coupling the DMTr group

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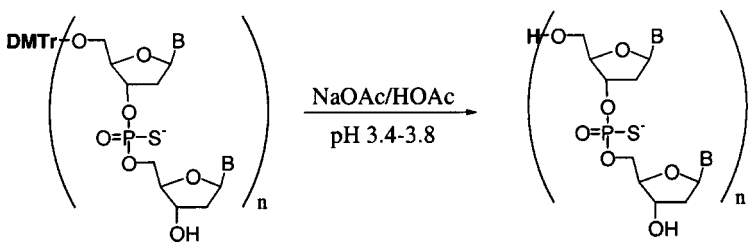
assures that only one amidite reacts with an oligonucleotide chain. Before the next amidite is coupled the DMTr group is removed by treatment with a solution of an organic acid in an organic solvent.[3] The hydrophobic DMTr group of the 5′-terminal nucleotide is left on the oligonucleotide to enable ready separation of full-length oligonucleotide (DMTr-on) from deletion sequences (DMTr-off).[4] Isolation of HPLC-purified oligonucleotide is followed by removal of the DMTr protecting group, typically with acetic acid.[4] It is well documented that oligodeoxyribonucleotides are prone to hydrolytic cleavage of the β-glycosidic linkage of purine nucleosides (deoxyguanosine, deoxyadenosine).[5] Depurination is accelerated at low pH, low ionic strength and elevated temperature.[6] The resulting apurinic oligonucleotide sequences exhibit lower binding affinity towards complementary oligonucleotide sequences.[7] Interactions of abasic sequences with oligonucleotides, proteins and intercalators have been reported. Protocols used for DMTr removal vary widely with respect to acid concentration and acid exposure time as well as work-up procedure. As a consequence, oligonucleotide products of varying quality are obtained among workers in the field. Measurement of the rate of DMTr removal from individual oligonucleotides under a well defined set of reaction parameters and calculation of the reaction time necessary for 'complete' removal of DMTr groups is, in our opinion, the key to achieving complete detritylation while keeping depurination at a minimum. In this report, we describe a simple procedure for controlled DMTr removal from synthetic oligonucleotides that reproducibly gives high quality products.

RESULTS AND DISCUSSION

Typical literature procedures recommend acidification of an oligonucleotide solution with highly concentrated acetic acid.[4] The pH of the reaction solution depends on amount and concentration of oligonucleotide and acetic acid. Oligonucleotide **DMTr-1** (1.2 mL, 200 OD_{260}/mL) was treated with acetic acid (75%, v = 50, 100 and 200 μL). The half life times for DMTr removal of this oligonucleotide with a 5′ terminal thymidine nucleotide at pH 2.97, 2.67 and 2.43 were 2 min, 1.3 min and 1 min, respectively. Reaction rates below pH 3 are rather high for efficient monitoring and adjustment of a desired pH is time consuming. In addition, acidification with highly concentrated acetic acid may lead to heterogenous reaction mixtures. Acidification of oligonucleotides in fractions directly obtained from RP-HPLC in the presence of methanol and sodium acetate requires large amounts of glacial acetic acid to



Scheme 1. DMTr removal.

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Table 1. Comparison of detritylation and depurination kinetics of phosphorothioate oligodeoxyribonucleotides **1**–**3** in NaOAc (10 mM).

Sequence	pH	$t_{1/2}$ [min] for DMTr removal	Depurination per hour [%]	Depurination per 15 $t_{1/2}$
PS-d(TCCCGCCTGT-GACATGCATT), **1**	3.56	8.1	0.43	0.87
PS-d(TCCGTCATCG-CTCCTCAGGG), **2**	3.54	5.8	0.58	0.84
PS-d(GTTCTCGCTG-GTGAGTTTCA), **3**	3.42	2.1	1.36	0.71

lower the pH. The buffer components slow down the detritylation reaction significantly. For example, DMTr removal from **1** at pH 3.9 in HPLC buffer (55% methanol, 0.2 M NaOAc) takes 15 h ($t_{1/2} = 1$ h) compared to 3.7 h ($t_{1/2} = 14.7$ min) when the reaction is performed at the same pH in NaOAc buffer (10 mM) of low ionic strength in the absence of organic solvent.

Using slightly acidic NaOAc solution of low ionic strength, it is possible to adjust the pH such that the DMTr removal reaction proceeds at an acceptable rate and can be monitored conveniently by reversed phase HPLC (see experimental). 3.5 volumes NaOAc buffer solution (10 mM, pH 3.0–3.2) added to one volume of oligonucleotide solution at a fixed concentration (1750 OD/mL) gives a pH of the reaction mixture in the range 3.4–3.8. Lower oligonucleotide concentration results in a lower pH. Reaction times of 0.5 to 2 h allow for sufficient time for HPLC analysis of the reaction mixture to calculate the appropriate reaction time. Assuming exponential decrease of DMTr-on species over time the half life for the reaction is calculated. The degree of 'completion' of DMTr removal depends on number of half lives. For most applications 15 half life times achieving 99.997% DMTr-off oligo may be considered sufficient. Table 1 shows half life times for DMTr removal of different PS-oligonucleotides in NaOAc buffer. We find different half life times for sequences with the same 5′ terminal nucleotide (for example **1** and **2**). The faster rate of sequence **3**, compared to **1** and **2**, is consistent with the observed order of detritylation rates during solid phase synthesis (dG > dA > dC > T).

Quantitation of free purine bases (adenine and guanine) by reversed phase HPLC is a very sensitive ($< 10^{-11}$ mol) method for following the progress of depurination. Oligonucleotides **1–3** were exposed to the final DMTr removal conditions. The formation of free adenine and guanine was linear over a 14 h period. De-adenylation is almost twice as fast as deguanylation. Table 1 compares the rates of depurination of **1–3** and the rate of final detritylation reaction in NaOAc buffer (10 mM). Depurination of **3** is 2–3 times faster than for **1** or **2**. However, detritylation of **3** proceeds also significantly faster than **1** or **2**. As a result, overall depurination of sequences **1**, **2** and **3** after 15 half life times of detritylation is almost identical, less than 1%.

Figure 1 shows a plot of residual DMTr-on oligo **1** over time and the formation of free purine bases which is equivalent to the formation of apurinic sequences. Furthermore the sum of both 'undesired' species is plotted. The minimum of this curve is between 10 and 11 detritylation half lives (this is also seen for **2** and **3**,

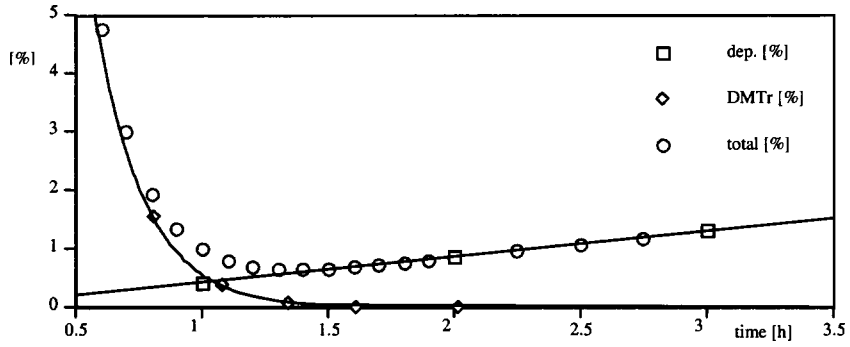

Figure 1.

data not shown). However, the curve around the minimum at longer reaction times is rather flat and extension of the detritylation time to 15 $t_{1/2}$ increases the amount of depurinated species only marginally while reducing the amount of DMTr-on species to ca 30 ppm. In summary, the method presented here allows complete DMTr removal while keeping depurination to a minimum.

EXPERIMENTAL PROCEDURE

Phosphorothioate oligodeoxyribonucleotides **DMTr-1-3** (Table 1) were synthesized using standard phosphoramidite coupling chemistry and 3*H*-1,2-benzodithiole-3-one-1,1-dioxide (Beaucage reagent) for sulfurization followed by cleavage from the solid support and deprotection of backbone and nucleobases with ammonium hydroxide (55°C, overnight). Subsequent DMTr-on/DMTr-off purification by RP-HPLC yielded fractions containing $>99.5\%$ DMTr-on oligonucleotide. The oligonucleotide was precipitated immediately by addition of ethanol (ca 4–5 volumes) to avoid detritylation prior to the actual detritylation step (calculation of $t_{1/2}$ is based on 100% DMTr-on oligonucleotide) and to remove salts and organic solvent. The oligonucleotide pellet was rinsed with ethanol and dried in vaccum.

DETRITYLATION EXPERIMENTS

DMTr-on oligonucleotide **DMTr-1-3** (350 $OD_{260\,nm}$) was dissolved in water (200 μL). Sodium acetate buffer (10 mM, pH adjusted to 3.0 with acetic acid, 700 μL) was added rapidly and the solution was vortexed for 5 s. After 5–10 min a sample (20 μL) was removed from the reaction mixture and added into aqueous sodium hydroxide (0.5 M, 1 mL). This solution (15 μL) was analyzed by RP-HPLC. The percentage of DMTr-on oligo was determined and the half life time for DMTr removal was calculated from the following equation:

$$t_{1/2} = \ln(0.5)/\ln(\%\mathrm{DMTr-on}/100) \times t \quad (1)$$

$$\text{Reaction time} = 15 \times t_{1/2} \quad (2)$$

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% DMTr-on = peak area DMTr-on/(peak area DMTr-on + peak area DMTr-off oligo) × 100, t = time from addition of NaOAc buffer to addition of reaction mixture into NaOH, $t_{1/2}$ = half life time for DMTr removal, reaction time = time from addition of NaOAc (pH 3.0) to time NaOAc (2.5 M) is added.

Upon completion of the reaction NaOAc (2.5 M, 30 μL) is added followed by ethanol (4 mL). The mixture is vortexed for 10 s and kept at –20°C for 15 min. The precipitated DNA is spun down by centrifugation. The supernatent is removed and the DNA pellet is rinsed with ethanol (1 mL). The DNA pellet is dried in vaccum.

DEPURINATION EXPERIMENTS

Oligonucleotides **1–3** (c = 350 OD/mL) were exposed to DMTr removal. At different time points (5 min, 1 h, 4 h, 8 h, 13 h) samples were analyzed by HPLC for the presence of free bases adenine and guanine. The amount of free base released was determined using the standard curve. The percentage of depurination of oligonucleotide was calculated by dividing the number of moles of base released by the number of moles of oligonucleotide injected. For quantitation of the oligonucleotides an extinction coefficient of ε = 165.000 was used.

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