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Controlled Detritylation of Antisense Oligonucleotides

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Abstract:
The last chemical step in standard oligonucleotide synthesis, the acid-catalyzed removal of 4,4′-dimethoxytrityl (DMTr) protecting groups from the 5′-terminus of oligonucleotides is accompanied by hydrolysis of purine nucleoside glycosidic linkages (depurination). A mild procedure for controlled DMTr removal using NaOAc (10 mM, pH 3.0) under a fixed set of conditions has been developed. The calculation of the reaction time is based on a $t_{1/2}$ versus pH profile, specifically determined for each individual oligonucleotide sequence. The effect of various solvent components on the reaction kinetics has been evaluated. As a result, oligonucleotides with improved impurity profile are obtained consistently from laboratory scale to production scale.

Introduction

Oligonucleotides have widespread application in diagnostics, molecular biology, and as therapeutic agents. DNA analogues, especially phosphorothioate oligonucleotides in which a nonbridging oxygen of the natural internucleotide phosphate group is replaced by sulfur, have emerged as potential drugs for treatment of diseases through an antisense mechanism of action.[1] Oligonucleotide analogues have attracted increasing attention as therapeutic agents especially after FDA approval of the first antisense oligonucleotide was recently achieved,[2] and many other antisense oligonucleotides are presently undergoing human clinical trials. These advances have necessitated the development of large-scale synthesis methods for the manufacture of therapeutic oligonucleotides.[3]

Automated synthesis of phosphorothioate oligodeoxyribonucleotides on solid support using a four-step synthesis cycle (detritylation, coupling, sulfurization, and capping) is best performed through amidite chemistry[4] using commercially available phosphoramidites **1a**−**d** (Scheme 1). The preferred protecting group for the 5′-hydroxyl of these starting materials is the 4,4′-dimethoxytrityl (DMTr) group. Removal of the acid-labile DMTr group from support-bound oligonucleotide plays a role in each cycle that is crucial to high product yield and quality.[5] After the assembly of the oligonucleotide is complete, the product is cleaved from the solid support and deprotected at the internucleotide linkages and the nucleobases. The hydrophobic DMTr group of the final 5′-nucleotide is retained to facilitate separation of full-length oligonucleotide ("DMTr-on") from shorter deletion sequences ("DMTr-off") by preparative reversed-phase high performance liquid chromatography (RP-HPLC). A final DMTr removal step post-purification produces the fully deprotected oligonucleotide. The major concern during this final step is lability of purine nucleotides in the acidic environment required for DMTr removal, leading to depurination as a side reaction[6] (Scheme 2). The resulting apurinic oligonucleotide sequences exhibit lower binding affinity towards complementary oligonucleotide sequences.[7] Even though methods for separation of apurinic sequences exist,[8] it is preferred to produce oligonucleotide consistently in high quality without additional purification steps.

DMTr removal from oligonucleotides is a standard operation on laboratory scale where the focus is on achieving complete deprotection quickly rather than minimizing depurination. The consequences of depurination of oligonucleotides with respect to performance of various assays is often overlooked but should not be ignored. Procedures for DMTr removal are typically very general. Oligonucleotide concentrations or reaction pH vary widely and fail to consider DMTr removal kinetics of individual oligonucleotides. Reported reaction times are typically not optimized and range from

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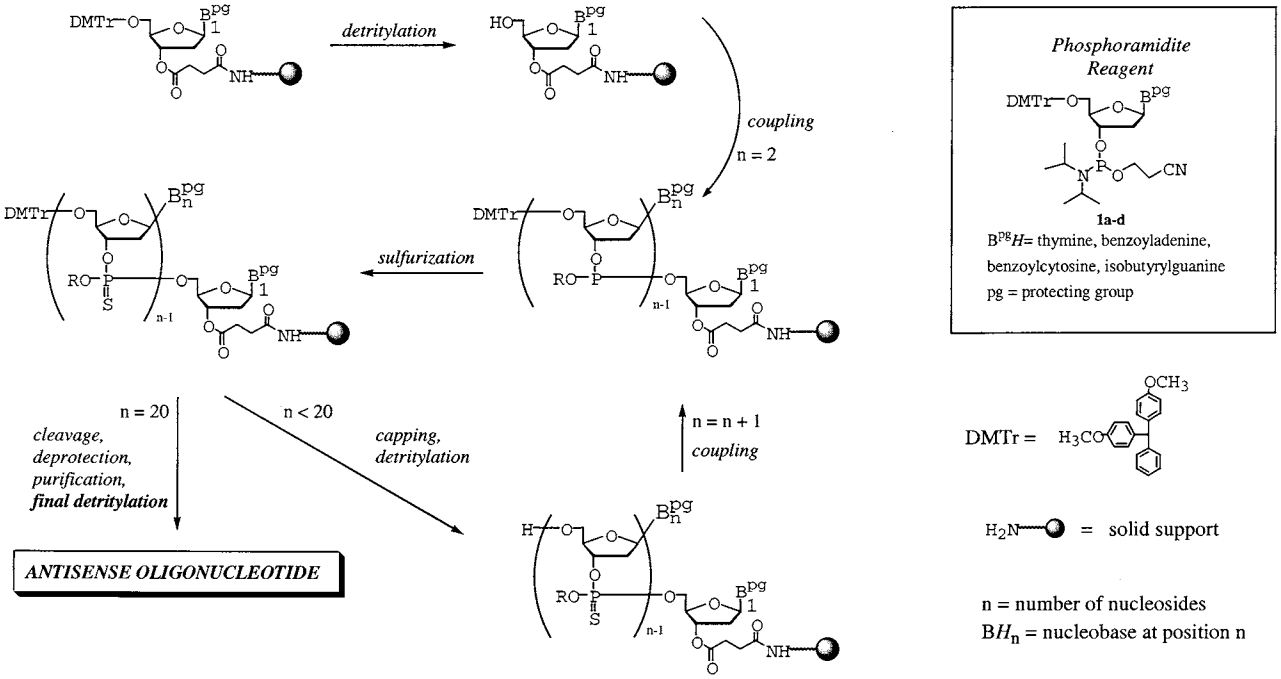
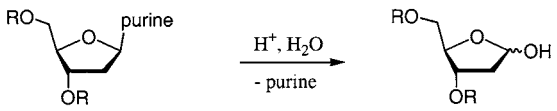
Scheme 1. **Chemical synthesis of phosphorothioate oligonucleotides**



Scheme 2. **Depurination**



purine = Gua, Ade

minutes up to one hour. Workup procedures such as evaporating solvent under vaccum at elevated temperature add additional acid exposure time and increase the level of apurinic sequences.

In continuation of our program directed to the development of improved methods for large-scale production of synthetic oligonucleotides, we were interested in optimizing this last chemical step of oligonucleotide synthesis. We find that for production of highest-purity oligonucleotides, complete DMTr removal with minimum depurination requires well-controlled reaction conditions optimized for each oligonucleotide. In this report, we describe mild procedures which can be applied for laboratory-scale as well as for large-scale removal of DMTr groups from oligonucleotides that allows batch-to-bath consistency and high quality.[9]

Results and Discussion

Both removal of DMTr groups and depurination are acid-catalyzed reactions. Within the constraints of conditions and reagents available for production of pharmaceutical agents we thought it would be rather difficult to effect detritylation without depurination. To limit formation of apurinic sequences to a minimum, the goal of this investigation was development of a practical procedure for DMTr removal of oligonucleotides on production scale with no more acid exposure than necessary for "complete" DMTr group removal. For the purpose of this investigation we define the DMTr removal reaction as "complete" when the DMTr-on oligonucleotide is well below a typical limit of quantitation by HPLC (0.1%). Since the DMTr-removal of oligonucleotides **2**−**4** can be described by a pseudo-first-order kinetics, we express reaction times in terms of half-lives ($t_{1/2}$). We consider acid exposure for 15 half-lives leading to 99.997% detritylated oligonucleotide as completion of the reaction. Process improvement focused on safe and optimized reaction conditions with the aim of large-scale synthesis reproducibly giving quality products for satisfactory therapeutical use.

Characteristics of Phosphorothioate Oligodeoxyribonucleotides 2−4. Compounds **2**−**4** (Table 1) are oligodeoxyribonucleotide 20-mers containing phosphorothioate internucleotide linkages for increased nuclease resistance instead of the natural phosphodiester linkages. The sequences are designed to inhibit translation of mRNA into proteins involved in various cancers through antisense mechanisms of action. **2** is a selective inhibitor of C-raf kinase used to inhibit growth of some solid tumors, including small cell lung carcinoma and breast cancer.[10] **3** is an inhibitor of Haras gene-expression.[11] **4** is a potent inhibitor of protein kinase C α gene expression.[10]

Synthesis of Phosphorothioate Oligodeoxyribonucleotides DMTr-2−4. Phosphorothioate oligodeoxyribonucleotides **DMTr-2**−**4** were synthesized using standard phosphoramidite-coupling chemistry and 3*H*-1,2-benzodithiol-3-

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Table 1. **Phosphorothioate oligodeoxyribonucleotides 2−4 used in this study**

cmpd	sequence	biological target
ISIS 5132 (**2**)	5′-PS-d[TCCCGCCTGTGACATGCATT]	c-*raf* kinase
ISIS 2503 (**3**)	5′-PS-d[TCCGTCATCGCTCCTCAGGG]	Ha-*ras*
Affinitac/ISIS 3521 (**4**)	5′-PS-d[GTTCTCGCTGGTGAGTTTCA]	PKC-α

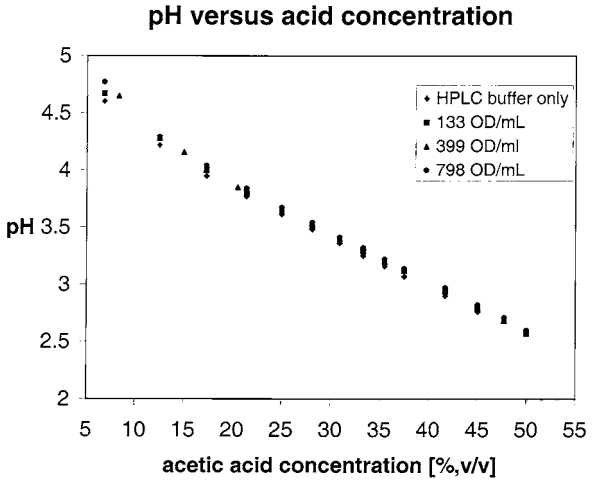

Figure 1. **Titration of phosphorothioate oligonucleotide 2 at different concentrations in HPLC buffer (45% methanol, 55% water, 0.18 M sodium acetate) with acetic acid.**

one-1,1-dioxide (Beaucage reagent) or phenylacetyl disulfide for sulfurization,[3] 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. HPLC-fractions were pooled and acidified, or the oligonucleotide was precipitated by addition of ethanol (ca. 4−5 vols) prior to the actual detritylation step to remove buffer salt and organic solvent.

DMTr Removal in HPLC Buffer. Acidification of the trityl eluate fractions with acetic acid appeared to be the shortest route to fully deprotected oligonucleotides. However, this process has some drawbacks. A significant amount of acetic acid required to lower the pH to a preparatively useful range is consumed by the buffer capacity of the salt (NaOAc) from the HPLC eluent, resulting in handling and consumption of large volumes of glacial acetic acid. To lower the pH to 3.5 ca. 25−30% glacial acetic acid (with respect to the volume of the trityl eluate) is required. Titration curves of oligonucleotide **2** at different concentrations (0, 133, 399 and 798 OD/mL) in HPLC buffer (55% water, 45% methanol, 0.18 M NaOAc) with 75% HOAc show that the pH is largely independent of the oligonucleotide concentration. (Figure 1) This may be considered an advantage in terms of reaction control and reproducibility. However, from an operational viewpoint the handling of large amounts of glacial acetic is less desirable. DMTr removal from oligonucleotide **2** in HPLC buffer is rather slow. At pH 3.5 the half-life time for DMTr removal from **DMTr-2** is 36 min, the total reaction time for complete DMTr removal is 9 h. It is evident that even more acetic acid is required to lower the pH further in the range were DMTr removal would occur at a faster rate.

Table 2. **Rate dependence of DMTr removal of DMTr-3 (15 OD/mL) as function of solvent composition**

entry	methanol [%, v/v]	NaOAc [M]	pH	$t_{1/2}$ [min]
1	0	0	4.18	10.3
2	4.5	0	5.10	35
3	22.5	0	5.01	35.5
4	45	0	5.10	73.9
5	52	0	3.50	10.4
6	0	0.009	3.24	2.9
7	0	0.009	3.81	5.2
8	0	0.009	3.85	6.0
9	4.5	0.009	3.80	5.6
10	9	0.009	3.77	5.5
11	22.5	0.009	3.73	6.2
12	45	0.009	3.50	7.9
13	45	0.009	3.57	9.5
14	40	0.036	3.08	14.6
15	0	0.045	3.22	3.9
16	0	0.045	3.70	7.4
17	45	0.045	3.54	12.9
18	36	0.066	3.09	20.5
19	32	0.12	3.11	28.5
20	0	0.18	3.25	9.0
21	0	0.18	3.56	10.2
22	4.5	0.18	3.56	12.4
23	9	0.18	3.53	13.9
24	22.5	0.18	3.53	24.3
25	45	0.18	3.52	39.4

We find that both HPLC buffer components, methanol and sodium acetate contribute to the slow reaction kinetics. An overview of various reaction conditions and the effects on rates of DMTr removal from **DMTr-3** is presented in Table 2. Under identical conditions the rate of detritylation increases as the pH of the reaction medium decreases, as expected (entries 6, 7, 8 and 15, 16 and 20, 21). Comparing DMTr removal at the same pH, but at different methanol concentrations (entries 2 and 4 and 21−25), we see a 2−4-fold increase in half-life as the methanol content is increased to 45%. At both low and high methanol concentrations we observe a ca. 3-fold increase in half-life time when the salt concentration (NaOAc) is increased to 0.18 M (entries 6, 15, 20 and 5, 13, 17, 25, respectively). Partial precipitation of some oligonucleotides at preparatively useful concentrations during acid addition limits further the general applicability of this procedure. Thus, we decided to look more closely at the parameters that determine the physicochemical aspects of this transformation.

DMTr Removal Using Aqueous Acetic Acid. In our first approach to overcome the drawbacks of the above method

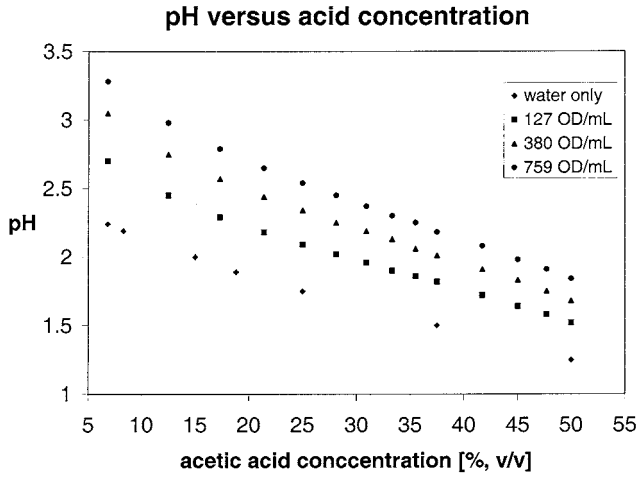

Figure 2. **Titration of aqueous solutions of phosphorothioate oligonucleotide 2 at different concentrations with acetic acid.**

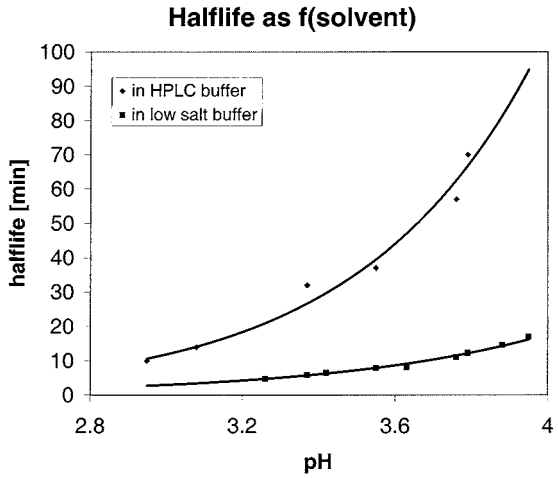

Figure 3. **Comparison of pH-rate profiles of DMTr removal of phosphorothioate oligonucleotides 2 in different media.**

we introduced an ethanol precipitation step of the oligonucleotide after HPLC purification, thus removing the HPLC buffer components, methanol and NaOAc, from the detritylation solution. The sodium salts of the oligonucleotides were then reconstituted in water. Typical literature procedures recommend acidification of an oligonucleotide solution with highly concentrated acetic acid. Titration curves of **2** in water show that the pH of the aqueous solution, in contrast to the HPLC buffer, depends on the oligonucleotide concentration. The higher the oligonucleotide concentration, the higher the pH at a given acid addition. (Figure 2) Oligonucleotide **DMTr-2** (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 at pH 2.97, 2.67, and 2.43 were 2, 1.3, and 1 min, respectively. Under those conditions, reaction rates below pH 3 are rather high for monitoring, thus making efficient reaction control a challenging task. Adjustment of a desired pH using concentrated acid is a critical step on larger scale when reaction vessels become quite large, especially when pH and timing become important issues. In addition, we find that addition of highly concentrated acid to more concentrated oligonucleotide solutions could lead to heterogeneous mixtures.

DMTr Removal with Low-pH NaOAc Buffer: Using HPLC for Monitoring. By using slightly acidic NaOAc solution of low ionic strength it is possible to adjust the pH of the detritylation reaction such that the DMTr removal reaction proceeds at an acceptable rate and can be monitored conveniently by RP-HPLC. In Figure 3 we compare reaction rates in HPLC buffer and in low-salt buffer. Rapid addition of 3.5 vols of NaOAc buffer solution (10 mM, pH 3.0−3.2) to 1 vol of oligonucleotide solution (c(**2**) = 1750 OD/mL) gives a pH of the reaction mixture in the range 3.5−3.8. Lower oligonucleotide concentration results in a lower pH. Total reaction time of 1−2 h allows enough time for HPLC analysis of the reaction mixture to calculate the appropriate reaction time based on a single time point. Assuming exponential decrease of DMTr-on species over time the half-life for the reaction may be 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 as sufficient. The faster rate of sequence **4**, compared to those of **2** and **3**, is consistent with the observed order of detritylation rates during solid-phase synthesis (dG > dA > dC > T). Although HPLC control of a reaction may not be very attractive in the routine manufacturing process of commercial products, this way of controlling the progress of a reaction has clearly some advantages. HPLC monitoring may be especially useful in settings where a large number of different oligonucleotides with unknown detritylation rates are processed. A higher success rate of completely removing the DMTr group and at the same time minimizing the competing process of depurination greatly improves the economics of the overall process and reduces losses. Further process-control improvements for commercial production of individual oligonucleotides are discussed in the following section.

DMTr Removal Using NaOAc Buffer: Using $t_{1/2}$ versus pH Profile. To optimize the process further and eliminate the need for HPLC monitoring during every detritylation reaction we determined the $t_{1/2}$ versus pH profile for oligonucleotides **DMTr-2**−**4** under a given set of conditions at lab scale. We fixed the oligonucleotide concentration (c = 1750 OD/mL) and volume (v_1) and the NaOAc buffer concentration (10 mM) and volume (v_2 = 3.5 v_1). The pH of the buffers, adjusted with acetic acid, ranged from 2.7 to 3.4 in 0.1 pH unit increments. We determined the half-life time for DMTr removal at each pH and generated a $t_{1/2}$ versus pH plot. (Figure 4) The data points were fitted by an exponential equation. Now, a simple pH measurement of the reaction mixture after acidification allows us to calculate the half-life using eqs 2−4 and the total reaction time. This process is scalable and we have demonstrated that data generated on laboratory-scale experiments is directly applicable to large-scale production. By keeping oligonucleotide concentration and buffer volume constant, the same pH is obtained in a reproducible manner from batch to batch, thus allowing the DMTr removal reaction to be performed without needing to measure the pH. This further demonstrates the robustness of this process. After typically 15 half-life

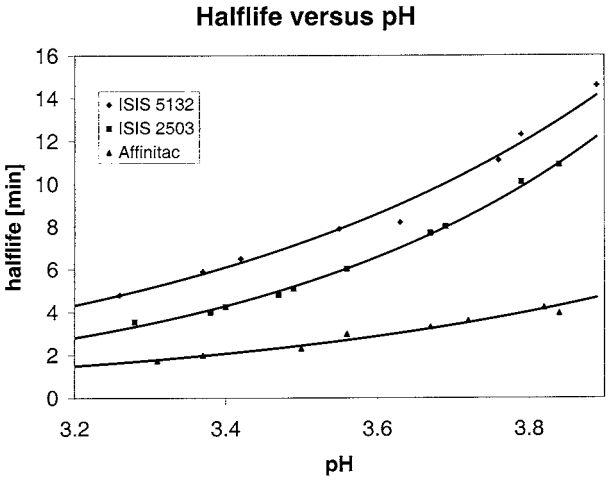

Figure 4. **Comparison of pH-rate profiles of DMTr removal of phosphorothioate oligonucleotides 2−4 using low-salt buffer conditions.**

Table 3. **Detritylation and depurination rates of phosphorothioate oligonucleotides 2−4**

cmpd	pH	-Gua [%][1]	-Ade [%][a]	total dep.[b]	$t_{1/2}$ [min][c]	total depurination [%][d]
2	3.56	0.044	0.084	0.43	8.1	0.87
3	3.54	0.071	0.11	0.58	5.8	0.84
4	3.42	0.14	0.26	1.36	2.1	0.73

[a] Per hour, normalized by the number of guanines or adenines present in the sequence. [b] Per hour. [c] Half-life time for DMTr removal [d] Depurination after complete detritylation (15 half-lives)

times, we raise the pH and precipitate the oligonucleotide by addition of ethanol. Temperature, another important reaction variable, was kept constant at 20 °C.

Depurination. Quantification of free purine bases (adenine and guanine) by analytical RP-HPLC is a very sensitive ($<10^{-11}$ mol) method for following the progress of depurination. Oligonucleotides **2**−**4** were exposed to the final DMTr removal conditions for an extended period of time (14 h). Table 3 compares the rates of depurination of **2**−**4** and the total amount of purines lost during the final detritylation reaction in NaOAc buffer (10 mM). The normalized percentage of purine loss per hour is the total amount of purine detected divided by the number of purine nucleotides present in each sequence (**2**: 4 dG, 3 dA; **3**: 5 dG, 2 dA; **4**: 6 dG, 2 dA). De-adenylation is almost twice as fast as de-guanylation. Depurination of **4** is 2−3 times faster than **2** or **3**. However, detritylation of **4** proceeds also significantly faster than **2** or **3**. As a result, overall depurination of sequences **2**, **3**, and **4** after 15 half-life times of detritylation is almost identical, less than 1%.

Conclusions

We have developed a practical, efficient, safer, and inexpensive procedure for large-scale removal of 4,4′-dimethoxytrityl groups from synthetic oligonucleotides in a controlled and reproducible manner. This straightforward process uses readily available reagents and provides major improvements over other methods previously described. The key features of this procedure are:

(1) precipitation of oligonucleotide from preparative HPLC fractions to remove buffer components, methanol and NaOAc, that slow the detritylation rate.

(2) performance of the detritylation reaction in aqueous medium using an acidic NaOAc buffer (10 mM) as reagent to reduce the amount of glacial acetic acid needed for efficient reaction kinetics. The reduced consumption of glacial acetic acid provides improvement in the operation of this part of the process. Higher reaction rates for DMTr removal in aqueous solution reduce the total downstream processing time significantly.

(3) generation of a $t_{1/2}$ versus pH curve (on lab scale) using the same experimental parameters (temperature, concentrations) used for large-scale production.

(4) measurement of the pH of the detritylation reaction at large scale and using the equation obtained under feature 3 above to calculate DMTr-ether half-life and required reaction time.

(5) use of constant reaction parameters to ensure reproducibility without the need for online reaction control.

(6) maximum detritylation at minimum acid exposure, to reduce depurination to a minimum. Resulting control of the reaction ensures batch-to-batch reproducibility.

Experimental Section

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*N*,*N*-diisopropylamino-*O*-(2-cyanoethyl) phosphoramidites (T, dA^{bz}, dC^{bz}, dG^{ib}) (Pharmacia, Sweden), 1*H*-tetrazole (American International Chemical, Natick), 3*H*-1,2-benzodithiol-one-1,1-dioxide (R.I. Chemical, Orange), phenylacetyl disulfide (Acharya, India), acetonitrile anhydrous (J. T. Baker), and toluene (Gallade) were used as received. Analysis and purification of oligonucleotides by RP-HPLC was performed on a Waters NovaPak C_{18} column (3.9 mm × 300 mm) using a Waters HPLC system (600E system controller, 996 photodiode array detector, 717 autosampler). For analysis an acetonitrile (A)/0.1 M triethylammonium acetate gradient was used: 5 to 35% A from 0 to 10 min, then 35 to 40% A from 10 to 20 min, then 40 to 95% A from 20 to 25 min, flow rate = 1.0 mL/min; for DMTr-on/DMTr-off purification: 5 to 27% A from 0 to 1 min, 27% A from 1 to 8 min, 27 to 50% A from 8 to 9 min, 9−26 min at 50%, flow rate = 1.0 mL/min, t_R(DMTr-off) 10−11 min, t_R(DMTr-on) 14−16 min.

***t*$_{1/2}$/pH Dependence.** Oligonucleotide **DMTr-2**−**4** (350 $OD_{260\ nm}$) was dissolved in water (200 μL). Sodium acetate buffer (10 mM, pH adjusted to 2.7−3.4 with glacial acetic acid, 700 μL) was added rapidly, and the solution was vortexed for 5 s. At different time points 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 oligonucleotide was determined, and the half-life time for DMTr removal was calculated from the following equation:

$$t_{1/2} = \left[\frac{\ln(0.5)}{\ln\left(\frac{\%\ \text{DMTr-on}}{100}\right)}\right] t \quad (1)$$

$$\%\ \text{DMTr-on} = \left[\frac{\text{peak area DMTr-on}}{(\text{peak area DMTr-on} + \text{peak area DMTr-off oligo})}\right] 100$$

t = time from addition of NaOAc buffer to addition of reaction mixture into NaOH

$t_{1/2}$ = half-life time for DMTr removal

The half-life times $t_{1/2}$ were plotted versus pH, and the best-fit exponential equation was determined. (Figure 4). The following equations were obtained:

$$\mathbf{2}:\ t_{1/2} = 0.0147 \times 10^{0.770} \quad (2)$$

$$\mathbf{3}:\ t_{1/2} = 0.0031 \times 10^{0.924x} \quad (3)$$

$$\mathbf{4}:\ t_{1/2} = 0.0074 \times 10^{0.72x} \quad (4)$$

x = pH of reaction

Large-Scale Detritylation of Oligonucleotide. Oligonucleotides are purified by RP-HPLC using a methanol/NaOAc gradient. Appropriate fractions containing DMTr-on oligonucleotide are pooled, and the oligonucleotide is precipitated with ethanol (5 vols). The precipitate is isolated by centrifugation and reconstituted in water (υ_1, c = 1750 OD/mL). At room temperature, a solution of NaOAc (υ_2 = 3.5υ_1, 10 mM, pH 3.0) is added to the oligonucleotide with agitation. After 5 min the pH is measured and the half-life time of the reaction is calculated using eqs 2−4. The total reaction time is 15 × $t_{1/2}$. After DMTr removal is complete NaOAc (υ_3 = 0.2 × υ_1, 3 M) is added, followed by ethanol addition. The oligonucleotide is isolated by centrifugation.

Depurination Experiments. Oligonucleotides **2**−**4** (c = 350 OD/mL) were exposed to the DMTr removal conditions (10 mM NaOAc, acetic acid). 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 (also thymine and cytosine). The amount of free base released was determined by using standard curves. 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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