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Synthesis of an Antisense Oligonucleotide Targeted against C-raf Kinase: Efficient Oligonucleotide Synthesis without Chlorinated Solvents

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Abstract—It is demonstrated that a solution of dichloroacetic acid in toluene removes dimethoxytrityl groups from the 5′-terminus of an antisense phosphorothioate oligodeoxyribonucleotide (ISIS 5132/CGP69846A) during synthesis on solid support cleanly and efficiently. It is therefore suggested to replace health hazardous dichloromethane which is typically used in oligonucleotide synthesis as solvent for DMTr-removal by toluene.

Introduction

Oligonucleotides are finding widespread utility in various applications in diagnostics and molecular biology and as therapeutic agents. In recent years, DNA analogues, especially phosphorothioate oligonucleotides in which one nonbridging oxygen atom of the internucleotide phosphate group is replaced by a sulfur atom, have emerged as potential drugs for treatment of a variety of diseases through antisense mechanisms of action.[1] ISIS 5132/CGP69846A (**3**) is a phosphorothioate oligodeoxyribonucleotide 20-mer [d(TCCCGCCTGTGACATGCATT)] targeted to the 3′-untranslated region of human C-*raf* mRNA, selectively inhibiting C-*raf* gene expression.[1c] **3** inhibits the growth of a variety of tumor types in vivo using nude mouse tumor xenografts.[1d] Sequence-specific antitumor activity supports an antisense mechanism of action in vivo.[1e] Clinical evaluation of **3** in humans as potential treatment for a variety of cancer targets is currently ongoing.

In standard synthesis of phosphorothioate oligodeoxyribonucleotides such as **3** through phosphoramidite coupling,[2] removal of the typical acid-labile 4,4′-dimethoxytrityl 5′-protecting group (DMTr), from the support-bound oligonucleotide plays a crucial role in each synthesis cycle in achieving high product yield and oligonucleotide quality.[3] Although several reagents have been developed for this purpose,[4] many have limited applicability to automated oligonucleotide synthesis on solid supports. The most commonly used reagents today are dilute solutions (2–15%) of an organic acid, typically trichloroacetic acid (TCA, pK_a 0.8) or dichloroacetic acid (DCA, pK_a 1.5) in dichloromethane. The high volatility (boiling point 40 °C) of dichloromethane and its high toxicity and carcinogenicity pose a hazard for personnel and the environment.[5] In addition, as oligonucleotide synthesizers are now available to allow syntheses of up to 0.2 mol scale,[6] the quantities of chlorinated waste generated have become quite large. In the context of potential ton-scale manufacturing of phosphorothioate oligonucleotides we became interested in replacing dichloromethane as deblocking reagent solvent with a less harmful solvent with preserving product yield and quality. It is necessary to keep deblocking conditions as mild as possible (low acid strength, short exposure times) in order to minimize side reactions such as depurination.[7] On the other hand, removal of the DMTr group in each cycle must be complete to avoid formation of DMTr-on deletion sequences [(n−1)-mers are particularly difficult to separate from the target sequence].[8] We now report that it is not necessary to use hazardous halogenated solvents such as dichloromethane in the deblocking step of automated oligonucleotide synthesis in order to obtain high yields of high quality oligonucleotide product.

Results and Discussion

In qualitative test tube experiments, several aliphatic solvents (DMF, hexanes, ethyl acetate, THF, *t*-butyl methyl ether) containing DCA (0.36 M) provided rather slow kinetics for removal of DMTr from DMTr-dC^{Bz} bound to controlled pore glass (CPG). In contrast, aromatic solvents like benzene, toluene, xylene, chlorobenzene and benzotrifluoride[9] led to rapid DMTr

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removal and therefore appeared to be candidates for replacement of dichloromethane. As initial screening, we performed a series of oligonucleotide syntheses (1 μmol) on an ABI 394 DNA/RNA synthesizer using commercially available DNA synthesis columns charged with CPG loaded with the 5′-*O*-DMTr-protected 3′-terminal nucleoside of the desired oligonucleotide. As test sequences we chose phosphorothioate oligodeoxyribonucleotides with a homo-T sequence T_{19} (**1**) [as thymidine has the slowest detritylation kinetics among the four protected nucleosides (DMTr-dG^{iBu} > DMTr-dA^{Bz} > DMTr-dC^{Bz} > DMTr-T)], a deoxyadenosine rich sequence $(TdAdA)_6T$ (**2**) (in order to compare the extent of depurination, as it is well known that benzoyl deoxyadenosine is prone to depurination[7]), and mixed-sequence phosphorothioate oligodeoxyribonucleotide **3**. We compared oligonucleotides synthesized with 0.36 M DCA (3%, v/v) or 0.18 M TCA (3%, w/v) in chlorobenzene, benzotrifluoride, benzene, toluene and xylenes versus syntheses using standard detritylation solutions at the same acid concentrations in dichloromethane. Dichloromethane for rinsing the solid support prior to detritylation was replaced by the same solvent used in the deblocking solution. During the deblocking step, detritylation solution (4 mL) was flushed through the column for 2 min. In each of the solvents, immediate occurrence of the typical orange-red color upon treatment with deblock solution indicated the immediate release of DMTr cations which allow for spectrophotometric tracking of each coupling step. Conductivity-based DMTr yields, which allow detection of major synthesis failures, were obtained when halogenated solvents like dichloromethane, trifluorotoluene or chlorobenzene were used. In contrast, no conductivity-based detritylation yield was obtained in less polar solvents like benzene, toluene, and xylenes (even though rapid DMTr removal took place as indicated by the orange color of the DMTr cation). The efficiency of the syntheses and the quality of the oligonucleotide product were judged by the full length content of crude and purified oligonucleotide product as well as by the relative amount of ($n-1$)-mer formed (as determined by capillary gel electrophoresis (CGE) of DMTr-off oligonucleotide). For purification, we used a C18 reversed-phase HPLC method that allowed facile separation of the 5′-DMTr-on oligonucleotide from the capped failure sequences. The total DMTr-on peak was collected without further fractionation. The results of the CGE analysis are summarized in Table 1. Full length contents of 86% for the homopyrimidine sequence **1** and 75 ± 1% for sequences **2** and **3** are typical when DCA (0.36 M) in dichloromethane is used for DMTr removal (entry 1). The relative ($n-1$)-mer content, defined as $(n-1)/[(n-1)+n] \times 100$, of 3.2 ± 0.3% in crude oligonucleotide product is due to incomplete coupling in the last coupling step and possibly incomplete detritylation during chain extension. Purification allows for separation of most of the shorter DMTr-off failure sequences, thus increasing the full length content to 93 ± 2% and reducing the relative ($n-1$)-mer content to < 2% for **1** and to 3 ± 0.5% for **2** and **3**. Using TCA (0.18 M, entry 2), similar results are obtained for the homopyrimidine sequence **1**, reduced full length content and increased ($n-1$)-mer content are observed for **2** and **3**, possibly due to depurination. Using DCA (0.36 M) in chlorobenzene, benzotrifluoride, benzene, toluene, xylene (entries 3, 4, 5, 6, 7) we obtained CGE traces of crude and purified oligonucleotide product almost identical to those when DCA/dichloromethane was used for DMTr removal. At a lower DCA concentration (0.18 M in toluene, entry 8), a slightly increased ($n-1$)-mer content is observed in sequence **1**. Surprisingly, very low full-length and very high ($n-1$)-mer contents in both crude and purified oligonucleotide products were obtained when TCA was used in any of the aromatic solvents listed in Table 1, as exemplified by the results obtained with toluene (entry 9). The high ($n-1$)-mer content seen in the DMTr-on fraction may be due to incomplete removal of the DMTr group. At higher acid concentration (0.36 M TCA in toluene, entry 10) the ($n-1$)-mer content was reduced, but was still unacceptable. As evident from the data presented in Table 1, a non-halogenated aromatic solvent like, for example, toluene allows efficient oligonucleotide synthesis. Halogenated aliphatic or aromatic solvents are not offering any advantage with respect to efficiency or purity of oligonucleotide products.

Solid support and reactor design play an important role in oligonucleotide synthesis. Currently, the preferred

Table 1. Full length content and relative ($n-1$)-mer content determined by CGE-analysis of phosphorothioate oligodeoxyribonucleotides T_{19} (**1**), $(TdAdA)_6T$ (**2**) and d(TCCCGCCTGTGACATGCATT) (**3**) synthesized on CPG support (1 μmol) using DCA or TCA in various solvents for DMTr removal

			Crude oligonucleotide			Purified oligonucleotide		
			$n-$mer [area-%]$\left(\frac{(n-1)}{n+(n-1)} \times 100\right)$					
Entry	Solvent	Acid, c[M]	1	2	3	1	2	3
1	CH_2Cl_2	DCA,0.36	86 (3.0)	76 (3.5)	74 (3.4)	95 (1.8)	91 (3.2)	93 (2.5)
2	CH_2Cl_2	TCA, 0.18	88 (2.7)	60 (6.8)	77 (4.6)	94 (1.7)	83 (5.3)	86 (3.5)
3	Chlorobenzene	DCA, 0.36	85 (2.8)	71 (4.7)	72 (4.1)	96 (1.5)	91 (2.8)	89 (2.8)
4	Benzotrifluoride	DCA, 0.36	86 (2.7)	69 (5.1)	73 (5.7)	93 (1.7)	90 (3.2)	92 (2.9)
5	Benzene	DCA, 0.36	85 (3.0)	71 (5.6)	77 (3.0)	96 (1.9)	92 (3.4)	91 (3.2)
6	Toluene	DCA, 0.36	87 (2.5)	73 (3.8)	79 (2.6)	96 (1.7)	90 (2.6)	90 (2.1)
7	Xylene	DCA, 0.36	85 (3.2)	72 (3.4)	73 (3.3)	95 (2.3)	91 (2.3)	90 (2.5)
8	Toluene	DCA, 0.18	89 (5.0)	76 (3.2)	75 (4.1)	89 (5.4)	91 (2.7)	90 (3.3)
9	Toluene	TCA, 0.18	43 (37)	54 (25)	47 (32)	40 (38)	58 (27)	55 (29)
10	Toluene	TCA, 0.36	73 (19)	39 (19)	42 (21)	72 (21)	73 (15)	73 (18)

support for large scale synthesis (100–200 mmol) is a polystyrene-based matrix (Primer Support, Pharmacia) which is tightly packed in a steel column. To simulate those conditions, we synthesized **3** on an OligoPilot I synthesizer (Pharmacia) in a packed bed column reactor (volume: 1 mL) using Primer Support-T (30 μmol scale) or CPG-T (20 μmol scale), comparing deblock solutions consisting of 0.36 M DCA or 0.18 M TCA in dichloromethane or toluene. In contrast to syntheses on the ABI synthesizer where a large amidite excess (8 equiv) is used, we limited the amount of amidite to 2.7 equiv (Primer Support) and 4 equiv (CPG) per coupling, respectively. For removal of the DMTr group, the deblock solution (20 mL) was flushed for 2 min through the column. CGE data of phosphorothioate oligonucleotide **3**, which was synthesized under all eight possible combinations of support, solvent and acid, are given in Table 2. Comparing the different solvent/acid combinations, we observed that the combination of toluene/DCA allows for synthesis of **3** in the same yield and with the same high degree of purity as DCA or TCA in dichloromethane. As observed in the 1 μmol experiments on the ABI synthesizer, the combination of toluene/TCA does not effect efficient detritylation under the conditions used, resulting in higher ($n-1$)-content in the crude oligonucleotide as well as in the HPLC-purified DMTr-on fraction. The number of crude ODs recovered from the syntheses is 134 ± 3 OD/μmol for CPG and 125 OD/μmol of Primer Support, independent of the deblock solution used. Again, these results demonstrate the high efficiency of DCA/toluene for removal of DMTr groups in oligonucleotide synthesis.

Detailed analysis of oligonucleotide **3** synthesized on 167 μmol scale in a packed bed reactor on Primer Support-T on an OligoPilot II synthesizer (Pharmacia) shows the equivalency of the oligonucleotide product from syntheses that used 0.36 M DCA in dichloromethane for deblocking and of 0.36 M DCA in toluene, xylenes or benzotrifluoride. Table 3 shows the analytical data and Figure 1 shows capillary gel electropherograms of crude and purified oligonucleotides. The yield of crude oligonucleotide (125 ± 2 OD/mg support) which was determined spectrophotometrically, the relative peak area of the DMTr-on fraction ($78 \pm 2\%$) in the reversed-phase HPL-chromatogram together with the full length content ($71 \pm 2\%$) from CGE analysis are indicative of efficient assembly of the oligonucleotide. The low relative ($n-1$)-mer contents in crude ($4.6 \pm 0.1\%$) and HPLC-purified oligonucleotide product ($2.4 \pm 0.1\%$) demonstrate very clearly that DMTr removal with DCA in aromatic solvents was as efficient as with DCA in dichloromethane. Negative ion mode electrospray mass spectroscopy confirmed the identity of the purified oligonucleotides. The phosphodiester content, determined by ^{31}P NMR spectroscopy or SAX HPLC,[10] was also within limits typically observed.

Chromatographic behavior in the deblocking step and acid binding to the oligonucleotide, which were described earlier for CPG-support,[3a] were also observed on Primer Support. More than two column volumes of deblock solutions must be passed through the column before the typical orange-red color of the DMTr cation is observed. However, it is important to note that a

Table 2. CGE-analysis of phosphorothioate oligodeoxyribonucleotide **3** synthesized on CPG (20 μmol) or Primer support (30 μmol) on an OligoPilot 1 synthesizer using DCA (0.36 M) or TCA (0.18 M) in dichloromethane or toluene for DMTr removal

Entry	Support	Acid	Solvent	Yield [OD/μmol]	Crude **3** $n-$mer [area%] $\left(\frac{(n-1)}{n+(n-1)} \times 100\right)$	Purified **3**
1	CPG	TCA	CH_2Cl_2	131	67 (6.7)	81 (6.2)
2	CPG	TCA	Toluene	133	58 (18)	67 (18)
3	CPG	DCA	CH_2Cl_2	131	72 (5.7)	80 (5.8)
4	CPG	DCA	Toluene	137	70 (5.7)	80 (5.7)
5	Primer	TCA	CH_2Cl_2	125	72 (4.4)	88 (3.0)
6	Primer	TCA	Toluene	125	65 (8.8)	85 (6.2)
7	Primer	DCA	CH_2Cl_2	125	74 (4.8)	87 (3.6)
8	Primer	DCA	Toluene	125	71 (4.8)	91 (3.0)

Table 3. Detailed analysis of phosphorothioate oligonucleotide **3** synthesized on Primer Support-T (167 μmol scale) using DCA (0.36 M) in various solvents

		Crude oligonucleotide					Purified oligonucelotide			
Entry	Solvent	Yield $[OD/\mu mol]_{260}$	DMTr-on [area%][a]	Full length content [%][b]	$\frac{(n-1)}{(n-1)+n}$	PO content[c] [%]	Full length content [%][b]	$\frac{(n-1)}{(n-1)+n}$	PS:PO:$(PO)_2$ ratio[d]	ES-MS[e] mol. mass
1	CH_2Cl_2	125	78 ± 2	72 ± 2	4.7%	0.60	93.7 ± 1	2.4%	87.9:10.7:1.4	6349.6
2	toluene	124	76 ± 2	69 ± 2	4.6%	0.85	94.9 ± 1	2.4%	83.7:14.2:2.1	6349.7
3	xylenes	125	80 ± 2	72 ± 2	4.6%	0.52	94.8 ± 1	2.4%	88.6:10.0:1.4	6349.7
4	benzotrifluoride	127	77 ± 2	69 ± 2	4.7%	0.78	93.0 ± 1	2.3%	86.7:11.6:1.7	6349.7

[a]By RP-HPLC ($\lambda = 254$ nm).
[b]By CGE ($\lambda = 260$ nm).
[c]By ^{31}P NMR.
[d]By SAX.
[e]Calculated mass = 6349.6.

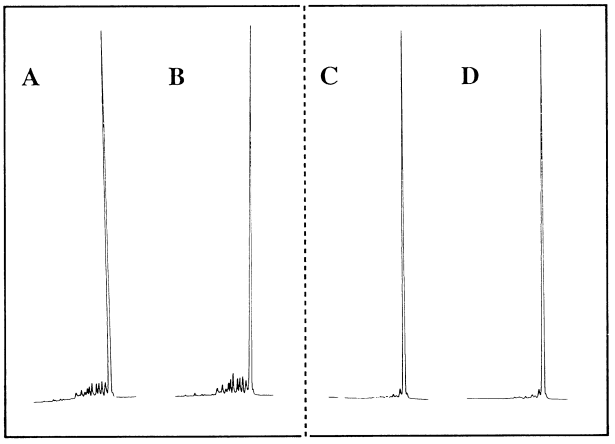

Figure 1. Comparison of CGE traces of **3** synthesized using 3% DCA/CH_2Cl_2 [crude (**A**), RP-HPLC purified (**C**)] or 3% DCA/toluene [crude (**B**), RP-HPLC purified (**D**)].

substantial quantity of DMTr groups are eluted in the first colorless fractions which are depleted of acid. Acid binding to the support and to the oligonucleotide are responsible for this delay. A plot of the time when the dimethoxytrityl absorption is first detected in the UV monitor versus base position shows a steady increase in detritylation start time with increasing number of nucleotides bound to the support (Fig. 2). From the difference in delay between the first and the 19th detritylation (the 20th nucleotide was synthesized DMTr-on) it may be estimated that on average ca. 2 mmoles of acid per mmole of nucleotide are absorbed.

Conclusion

As evident from the data presented in this paper, a solution of dichloroacetic acid in toluene allows removal of dimethoxytrityl groups from support-bound oligonucleotides providing antisense oligonucleotide ISIS 5132/CGP69846A (**3**) in high yield and purity. Multiple analytical methods showed equivalence to oligonucleotides synthesized with the standard solution of dichloroacetic acid in dichloromethane. In conclusion, we have shown that health hazardous dichloromethane as solvent for deblocking in solid phase synthesis can be replaced by toluene, without compromising yield or quality of the oligonucleotide products.[11]

Experimental

The ^{31}P NMR spectra were recorded on a Unity-400 spectrometer (Varian) operating at 161.9 MHz. Strong anion exchange high performance liquid chromatography (SAX-HPLC) of DMTr-off oligonucleotides was performed on a Resource Q column (1 mL) from Pharmacia, temp 60 °C, flow rate 1 mL/min, $\lambda = 260$ nm, mobile phases: NaCI (1 M), sodium phosphate (0.1 M) pH 11.5 (A), NaCI (2 M), sodium phosphate (0.1 M) pH 11.5 (B), gradient: 100% A for 5 min, 100% to 0% A from 5 to 40 min, t_R(all-PS) 27.5 min, t_R(mono-PO) 24.7 min, t_R(diPO) 22.1 min. Capillary gel electrophoresis was performed on a eCAP ssDNA 100 Gel Capillary (47 cm) on a P/ACE System 5000 using tris/borate/7M urea buffer (all Beckman), running voltage 14.1 kV, temp 40 °C. Electrospray ionization mass spectrometry was performed using a Hewlett–Packard 59987A electrospray quadrupole mass analyzer using negative polarity.

Phosphorothioate oligodeoxyribonucleotide synthesis

(a) Applied Biosystems 394 DNA/RNA synthesizer, commercial columns with CPG-T support (loading: 44 μmol/g, Glen Research), scale: 1 μmol, standard 5′-*O*-DMTr phosphoramidites (0.1 M in CH_3CN), 1*H*-tetrazole (0.45 M in CH_3CN), coupling time: 25 s, 3*H*-1,2-

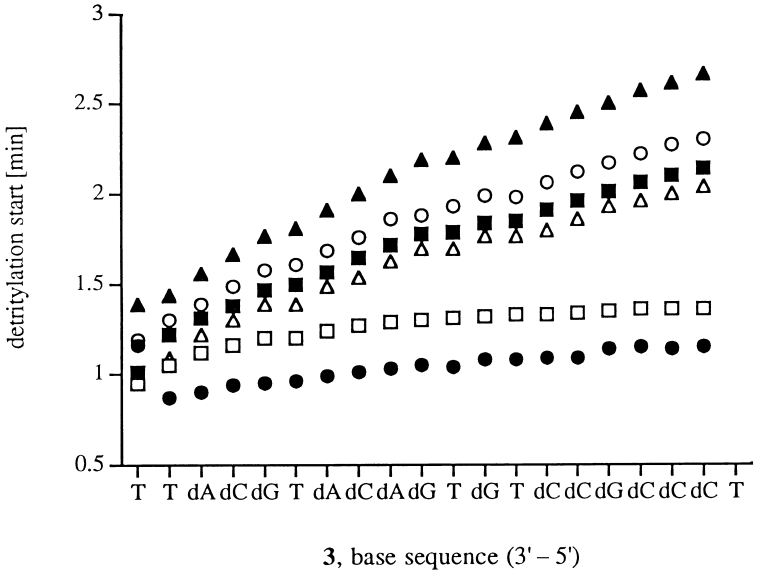

Figure 2. Detritylation start time versus nucleotide position on oligonucleotide **3**. 3% DCA/benzotrifluoride (▲), 3% DCA/xylenes (○), 3% DCA/toluene (■), 3% DCA/CH_2,$C1_2$ (Δ), 5% DCA/toluene (□) 10% DCA/toluene (●).

benzodithiol-3-one-1,1-dioxide (0.2 M in CH_3CN) for 300 s. (b) Pharmacia OligoPilot I, CPG-T support (loading: 49.6 μmol/g, Perseptive Biosystems), scale: 20 μmol, Primer-T support (loading: 88 μmol/g, Pharmacia), scale: 30 μmol, standard 5′-*O*-DMTr phosphoramidites (0.2 M in CH_3CN), 1*H*-tetrazole (0.45 M in CH_3CN), coupling time: 3 min, 3*H*-1,2-benzodithiol-3-one-1,1-dioxide (0.5 M in CH_3CN) for 1.2 min. (c) Pharmacia OligoPilot II, Primer-T support (loading: 88 μmol/g), scale: 167 μmol, standard 5′-*O*-DMTr phosphoramidites (2 equiv, 0.2 M in CH_3CN), 1*H*-tetrazole (0.45 M in CH_3CN), coupling time: 5 min recycling, 3*H*-1,2-benzodithiol-3-one-1,1-dioxide (4.5 mL, 0.5 M in CH_3CN) for 30 s.

Workup

Primer support (ca. 100 mg) was treated with 2 mL NH_4OH (30%) for 15 h at 60 °C, filtered, rinsed with ethanol/water (1/1,v/v), and the combined solutions were evaporated to dryness under vacuum. CPG support (ca. 150 mg) was treated with 2 mL NH_4OH (30%) for 1 h at room temperature, filtered, rinsed with 1 mL NH_4OH (30%) and the combined solutions were kept at 60 °C for 15 h, followed by evaporation to dryness under vacuum. The residue was dissolved in 200 μL water.

Dedimethoxytritylation

An aliquot (30 μL) was transferred into an Eppendorff tube (1.5 mL), and acetic acid (50%, 30 μL) was added. After 30 min at room temperature sodium acetate (2.5 M, 20 μL) was added, followed by cold ethanol (1.2 mL). The mixture was vortexed and cooled in dry ice for 20 min. The precipitate was spun down with a centrifuge, the supernatent was discarded and the precipitate was rinsed with ethanol and dried under vacuum.

HPLC analysis and purification

Analysis and purification of oligonucleotides by reversed-phase high performance liquid chromatography (RP-HPLC) was performed on a Waters Nova-Pak C_{18} column (3.9×300 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/50% A from 8 to 9 min, 9 to 26 min at 50%, flow rate = 1.0 mL/min, t_R(DMTr-off) 10–11 min, t_R(DMTr-on) 14–16 min. The DMTr-on fraction was collected and was evaporated in vacuum, redissolved in 50 μL water and the DMTr group was removed as described above.

ES/MS sample preparation

HPLC-purified and dedimethoxytritylated oligonucleotide was dissolved in 50 μL water, ammonium acetate (10 M, 5 μL) and ethanol were added and vortexed. The mixture was cooled in dry ice for 20 min and after centriguation the precipitate was isolated. This procedure was repeated two more times to convert the oligonucleotide to the ammonium form. The oligonucleotide was redissolved in water/iso-propanol (1/1, 300 μL) and piperidine (10 μL) was added.

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