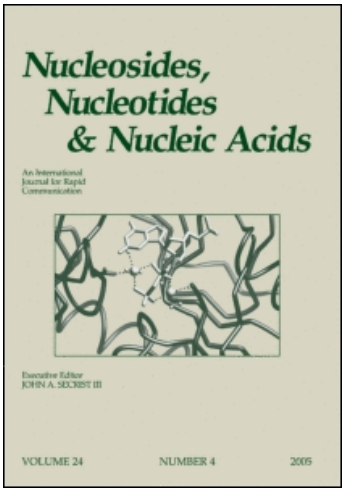

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Biologically Active Antisense Phosphorothioate Oligodeoxyribonucleotides: Synthesis, Characterization, and Studies of 3′-Terminal Phosphorothioate Monoester Analogues[†]

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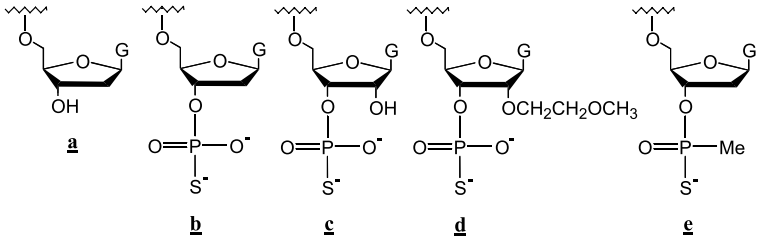
ABSTRACT

Multiple phosphorothioate oligonucleotides containing a 3′-terminal negative charge were synthesized and characterized. Influence of the added negative charge on activation of duplexes by RNase H was investigated. No additional help in recruitment of RNase H was observed.



Key Words: Antisense phosphorothioate oligonucleotides; 3′-TPT; RNase H activation.

[†]In honor and celebration of the 70th birthday of Professor Leroy B. Townsend.

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INTRODUCTION

Antisense oligonucleotides are designed to hybridize to specific regions of a selected mRNA in order to interfere with normal processing of the mRNA, leading to reduced expression of the coded protein.[1–7] These molecules are believed to function predominantly through two mechanisms, 1) translational arrest and 2) destruction of mRNA by ribonuclease H (RNase H).[8] While both mechanisms can successfully limit protein production, facilitated RNase H cleavage of target mRNA is considered to be the more efficient. Since a wild-type phosphate diester backbone is rapidly degraded by nucleases, several modifications have been investigated to increase antisense drug stability and target affinity. RNase H is a ubiquitous enzyme that specifically recognizes and hydrolyzes the RNA strand of an RNA-DNA duplex.[9–13] It is now well established that modifications that lead preferentially to a Northern-type sugar puckering (including its constrained form) in an RNA-type antisense oligonucleotide result in loss of RNase H activity, because they resemble an RNA/RNA duplex. Oligonucleotide-assisted RNase H-dependent reduction of targeted RNA expression can be quite efficient, reaching 80-95% down-regulation of protein and mRNA expression. Thus, the most widely used molecule is a chimeric phosphorothioate oligonucleotide consisting of 2′-*O*-alkyl (preferably 2′-*O*-methoxyethyl) RNA flanking a central core segment of DNA.[14–17] This design provides good RNA binding affinity via the wings and recruitment of RNase H for degradation of target RNA via the gap DNA core.

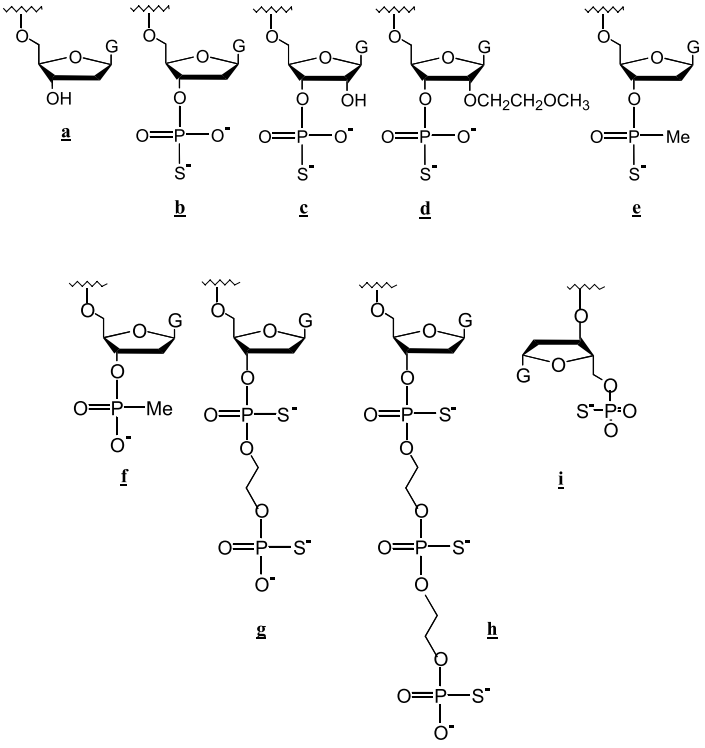

Figure 1. 3′-modifications synthesized for evaluation.

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Table 1. Obligonucleotides used for the investigation.

Oligo #	Sequence	3′-terminal modification (G_x)
1	PS[d(CTA-CGC-TTT-CCA-CGC-ACA-G)]	a
2		b
3		c
4		d
5	PS[d(CTA-CGC-TTT-CCA-CGC-ACA-G_x)]	e
6		f
7		g
8		h
9		i

Several antisense drugs based on this motif are in clinical trials for a range of diseases including arthritis and adult onset diabetes.[a] However, the factors governing recruitment of RNase H to target cleavage are not fully understood. In order to increase the therapeutic value of antisense drugs further investigation is essential.

Medicinal chemistry directed to increasing therapeutic potency of antisense drugs has addressed modified internucleotide linkers, heterocyclic bases, and sugar moieties. Several reporter groups,[18–20] including high molecular weight polyethylene glycol[21] have been attached at the 3′-terminal end of oligonucleotides and their influence on RNase H activity have been reported. In addition, conformation-specific cleavage of antisense-RNA duplexes by RNase H has been reported.[22] However, the ability of hybrid complexes formed by oligodeoxyribonucleotides containing a 3′-terminal negative charge to activate RNase H has not been extensively studied even though some studies have been reported.[23,24]

As part of our broad program to design antisense molecules to enhance recruitment of RNase H, we were interested in investigating the influence of negative charge at the 3′-terminal on the biological activity of antisense molecules. In this work, we studied the effect of various 3′-pendant negative charges on RNase H-induced RNA hydrolysis in hybrid duplexes.

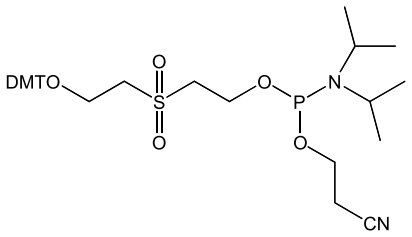
RESULTS AND DISCUSSION

Negative charge can be introduced in the forms of phosphate, carboxylate or other functionalities. In the present study we chose to investigate phosphate and its analogs. Thus, phosphate, phosphorothioate, methyl phosphonate, and methyl phosphorothionate were selected for investigation. A 19-mer phosphorothioate oligodeoxyribonucleotide targeted to BCLx expression inhibition was chosen as the antisense oligonucleotide. At the 3′-end of the oligomer several modifications carrying negative charge (Figure 1**b**–**i**), along with a control molecule (**a**), were synthesized and evaluated (Table 1).

[a]Multiple second-generation phosphorothioate oligonucleotides are in various stages of evaluation targeted against TNFα, VLA4, C-*raf*, PTB1B, ApoB 100, etc for a variety of diseases such as cancer, asthma, arthritis, multiple sclerosis, etc.

Synthesis and Characterization of Oligonucleotides Containing Modifications

Introduction of a phosphorous center negative charge at 3′-terminal of an oligonucleotide can be effected in several ways.[25–38] Although commercially available glass supports (CPG) containing Phosphate-ON reagent are available, the corresponding version of Primer Support PS200 is not commercially available.



We decided therefore to use the standard thymidine-loaded PS200 Phosphate-ON reagent solid support and couple a Phosphate-ON phosphoramidite first, then proceed with oligonucleotide synthesis. Afterward, incubation with concentrated aqueous ammonium hydroxide liberates the phosphorothioate oligonucleotide from the support with formation of the thymidine-5′-phosphate monoester. This nucleoside monomer is easily removed during reverse phase HPLC purification. In addition, we recently reported a novel approach to synthesis of 3′-phosphate/phosphorothioate monoester utilizing a ''trimethyl lock'' based molecule. This derivatized solid support was successfully used in synthesis of several 3′-negatively charged oligonucleotides (Figure 2).[39]

Crude DMT-on oligomer was purified by reverse phase HPLC under standard conditions, fractionated and the desired fractions were pooled. Detritylation was performed in the usual way, and the oligomer was precipitated and lyophilized to afford a colorless amorphous powder. The purified oligonucleotide was analyzed by capillary gel electrophoresis (CGE) (Figure 3),[30] P NMR (Figure 4) and electrospray quadrupole mass spectroscopy (Table 2).

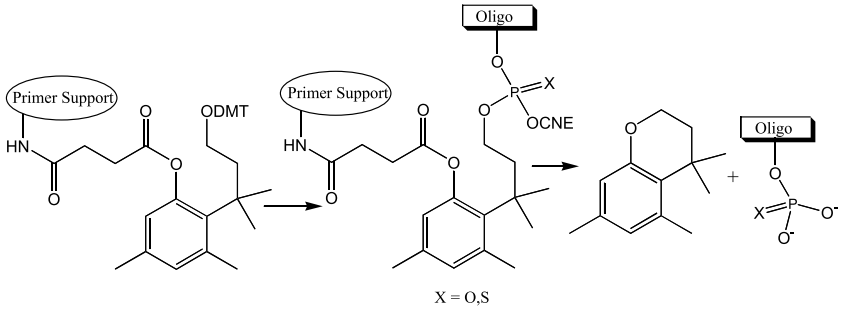


Figure 2. Novel solid support used for synthesis of 3′-TPT.

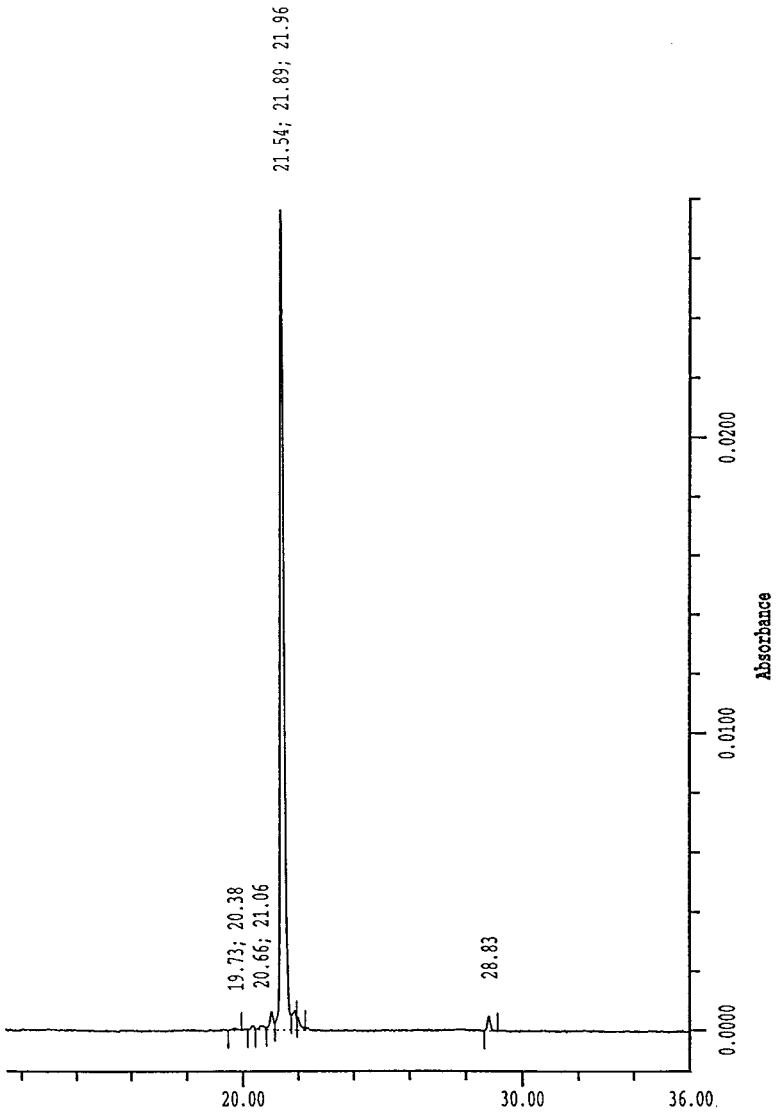


Figure 3. CGE analysis of phosphorothioate oligonucleotide (3g).

RNase H Initial Rate Determination for Duplexes Formed with Oligonucleotides Containing 3′-Modifications

^{32}P Labeling of Oligoribonucleotides

The sense strand was 5′-end labeled with ^{32}P using [γ-^{32}P]ATP, T4 polynucleotide kinase, and standard procedures.[40] The labeled RNA was purified by electrophoresis

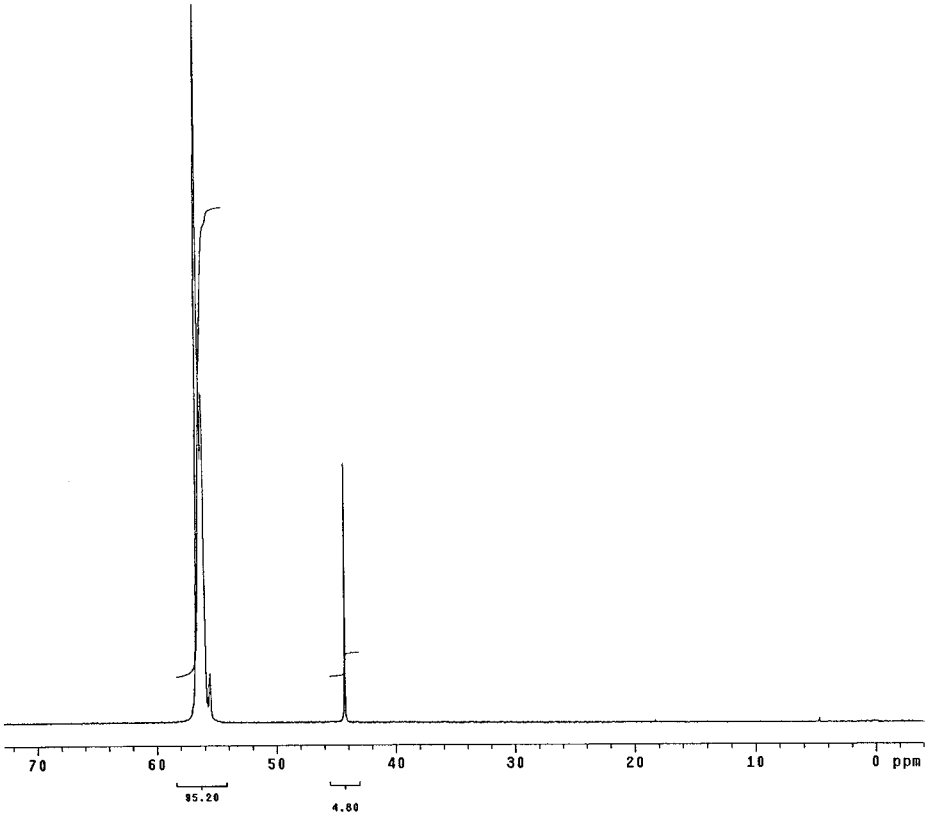


Figure 4. ^{31}P NMR (D_2O) of phosphorothioate oligonucleotide (3g).

on 12% denaturing PAGE. The specific activity of the labeled oligonucleotide was approximately 6000 cpm/fmol.

Determination of Initial Rates

Hybridization reactions were prepared in 100 μL of reaction buffer [20 mM tris, pH 7.5, 20 mM KCl, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol] containing 100 nM antisense phosphorothioate oligonucleotide, 50 nM sense oligoribonucleotide, and 100,000 CPM of ^{32}P labeled sense oligoribonucleotide. Reactions were heated at 90°C for 5 min. and cooled to 37°C prior to adding $MgCl_2$. Hybridization reactions were incubated overnight at 37°C. Hybrids were digested with 0.5 ng human RNase H1 at 37°C.[41] Digestion reactions were analyzed at specific time points in 3 M urea and 20 nM EDTA. Samples were analyzed by trichloroacetic acid assay.[42] The concentration of substrate converted to product was plotted as a function of time. The initial cleavage rate (V_o) was obtained from the slope (pM converted substrate per minute) of the best-fit line derived from ≥ 5 data points within the linear portion (< 10% of the total

Table 2. Characteristics of DNA analogues possessing 3′-terminal charge.

Oligo #	HPLC retention time, min.[a]	Mass	
		Calculated	Found
1	21.02	5997.20	5997.26
2	20.94	6093.82	6093.37
3	20.94	6108.97	6109.34
4	20.96	6167.72	6167.41
5	21.05	6090.89	6091.43
6	20.88	6075.13	6075.30
7	20.97	6231.95	6232.36
8	20.94	6371.88	6371.36
9	20.91	6093.22	6093.71

[a]Phenomenex, C18, 4.6 × 250 mm, A = 100 mM triehylammonium acetate, pH 7, flow rate 1.0 mL min^{-1}, λ = 260 nm, B = acetonitrile, 0–4% B from 0 to 25 min, 40% B from 25 to 30 min, 100% B from 30 to 39 min, 100% A from 39 min to 45 min.

reaction) of the plot.[43] The errors reported were based on three trials and is shown in Table 3.

CONCLUSION

Under the reported conditions, 3′-TPT and their analogs do not affect the structure of the hybrid duplex nor the consequent RNase H activity; thus these derivatives stimulate the hydrolysis of RNA by the enzyme at the same site and with the similar extent of cleavage as the native sequence. From these data it is not possible to recognize any significant difference between phosphate/phosphorothioate and phosphonate/phosphonothioate. In summary, synthesis of various phosphorothioate oligodeoxyribonucleotides containing negative charge at 3′-terminal were synthesized and characterized.

Table 3. Rate of cleavage of duplex formed with oligonucleotides containing modifications.

Oligo #	V_0 (pM/min)	
1	869 ± 0.953	–
2	850 ± 0.965	0.728
3	564 ± 0.937	0.009
4	569 ± 0.936	0.008
5	1016 ± 0.966	0.201
6	982 ± 0.944	0.264
7	813 ± 0.963	0.049
8	793 ± 0.955	0.002
9	792 ± 0.935	0.012

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RNase H initial rate determination on the duplexes formed with these oligonucleotides containing modifications was determined.

EXPERIMENTAL

Anhydrous acetonitrile (water content < 0.001%) was purchased from Burdick and Jackson (Muskegon, MI). 5′-*O*-Dimethoxytrityl-3′-*N*,*N*-diisopropylamino-3′-*O*-(2-cyanoethyl)phosphoramidites (T, dA^{bz}, dC^{bz}, dG^{ibu}) were purchased from Amersham Pharmacia Biotech, Milwaukee, WI. Methyl phosphonamidite, ethylene glycol amidite, inverted amidite and Phosphate-ON reagent were purchased from ChemGenes, MA. Toluene was purchased from Gallade, Escondido, CA. All other reagents and dry solvents were purchased from Aldrich and used without further purification. Primer support PS200 was purchased from Amersham Pharmacia Biotech, Uppsala, Sweden. 1*H*-Tetrazole was purchased from American International Chemical, Natick, MA. Phenylacetyl disulfide (PADS) was purchased from H. C. Brown Laboratories, Mumbai, India.

^{31}P NMR spectra were recorded on a Unity-200 spectrometer (Varian, Palo Alto, CA) operating at 80.950 MHz. Capillary gel electrophoresis was performed on a eCAP ssDNA 100 Gel Capillary (47 cm) on a P/ACE System 5000 using Tris/borate/7 M urea buffer (all Beckman), running voltage 14.1 kV, temperature 40°C. For synthesis of 3, the support-bound DMT-on oligonucleotide was first treated with triethylamine:acetonitrile (1:1, v/v) at room temperature for 8 h, then treated with Et_3N-3HF for 7 h at room temperature and then incubated with ammonium hydroxide in the usual manner.

Solid Supported Synthesis of Oligonucleotides

All syntheses were performed on a Pharmacia OligoPilot II DNA/RNA synthesizer using β-cyanoethyl phosphoramidite synthons (2.5 equivalents, 0.2M in CH_3CN). 1*H*-Tetrazole (0.45M in CH_3CN) was used as activator and phenylacetyl disulfide (PADS) (0.2M in 3-picoline-CH_3CN 1:1, v/v) as sulfur transfer reagent[44,45] Capping reagents were made to the recommended Pharmacia recipe: Cap A: *N*-methylimidazole-CH_3CN (1:4 v/v), Cap B: acetic anhydride-pyridine-CH_3CN (2:3:5, v/v/v). Pharmacia HL30 T Primer support (loading 94 μmole/gram) was used in all experiments. Amidite and tetrazole solutions were prepared using anhydrous CH_3CN (ca 10 ppm) and were dried further by addition of activated 4 Å molecular sieves (∼ 50 g/l). 5′-Phosphate-ON reagent was used as a 0.2M solution (2.0 equivalents) in CH_3CN. To introduce the 3′-terminal charge, the commercially available 5′-phosphate-ON reagent was first coupled to the T Primer solid support, then the oligonucleotide constructed. At the end of each synthesis, DMT-on oligonucleotide bound to support was transferred to a 500 ml pyrex glass bottle and treated with CH_3CN:Et_3N (1:1, v/v, 400 ml) at room temperature overnight. The support was filtered and taken up in a 250 ml Pyrex glass bottle. Concentrated aqueous ammonium hydroxide (400 ml) was added and incubated in an oven at 55°C for 18 h. The bottle was then cooled to room temperature and the solid filtered on a sintered glass funnel. The support was washed with water (250 ml), the combined solution concentrated by rotary evaporator. Triethylamine (4 ml) was added and the product was stored in a refrigerator. Details of the synthesis cycle are given in Table 4.

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Table 4. Synthesis parameters of cycle used on Pharmacia OligoPilot II synthesizer.

Step	Reagent	Volume (ml)	Time (min)
Detritylation	10% dichloroacetic acid/toluene	72	1.5
Coupling	Phosphoramidite (0.2M), 1*H*-tetrazole (0.45 M) in acetonitrile	10, 15	5
Sulfurization	Phenylacetyl disulfide (0.2M) in 3-picoline-CH_3CN (1:1, v/v)	36	3
Capping	Ac_2O/pyridine/CH_3CN, NMI/CH_3CN	24, 24	2

HPLC Analysis and Purification of Oligonucleotides

Analysis and purification of oligonucleotides by reversed phase high performance liquid chromatography (RP-HPLC) was performed on a Waters Novapak 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)/0.1M 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% A, flow rate = 1.0 mL/min, t_R(DMT-off) 10-11 min, t_R(DMT-on) 14-16 min. The DMT-on fraction was collected and was evaporated in vacuum, redissolved in water and the DMT group was removed as described below.

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 (2.5M, 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 on a centrifuge, the supernatant was discarded and the precipitate was rinsed with ethanol and dried under vacuum.

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 centrifugation 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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