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Antisense Phosphorothioate Oligodeoxyribonucleotide Targeted against ICAM-1: Synthetic and Biological Characterization of a Process-Related Impurity Formed During Oligonucleotide Synthesis

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Abstract—A phosphorothioate-linked oligonucleotide bearing a 3′-terminal phosphorothioate monoester has been synthesized and characterized. This oligonucleotide has been identified as a process-related impurity formed during synthesis of ISIS 2302. Biological properties of the compound have been determined. Based on these data, it can be concluded that this species (3′-TPT) has biological properties similar to parent drug.

Introduction

The concept of treating disease with antisense oligonucleotides became reality with approval of Vitravene™. The large number of antisense drugs in clinical trials and future market demand for systemic antisense oligonucleotides have stimulated efforts toward developing cost-efficient large-scale syntheses of this new class of drugs. In addition, efforts are being made toward improving quality of these complex molecules. These efforts have led to routine synthesis of 20-mer oligodeoxyribonucleotide phosphorothioate at 300–600 mmol run scale using only 1.75-fold molar amidite excess in less than 7 h total synthesis time.[1]

Quality of Phosphorothioate Oligodeoxyribonucleotides

Quality of the final active pharmaceutical ingredient (API) being synthesized at Isis Pharmaceuticals, Inc. has been markedly improved as compared to batches just a few years ago. This is due mainly to three factors: control of raw material quality, improved oligomerization efficiency, and side reaction reduction. We now understand how detectable process-related impurities are formed and eliminated or substantially reduced most of their levels, thereby markedly improving oligonucleotide quality. In addition, the analytical tools (capillary gel electrophoresis, anion exchange HPLC, PAGE, etc.) used earlier have been recently replaced by IP-LC–MS which is capable of speciating and quantifying all process-related impurities in these class of drugs.

Synthesis of ISIS 2302

Syntheses of ISIS 2302 (**1**, 5′-GCC-CAA-GCT-GGC-ATC-CGT-CA) are routinely being performed on an Amhersham-Pharmacia Biotech OligoProcess at 400–600 mmol scale using optimized conditions leading to high yields and quality as compared to conventional processes.

During the synthesis and analytical control of phosphorothioate oligonucleotide **1** for clinical evaluation, we observed by capillary gel electrophoresis (CGE) of purified active pharmaceutical ingredient a peak almost equal in area-percent to that of (n-1)-mers but migrating more rapidly (Fig. 1). We reasoned that it was not an (n-2)- or an (n-3)-mer. Isolation of the unknown by slab gel electrophoresis and characterization by LC–MS revealed

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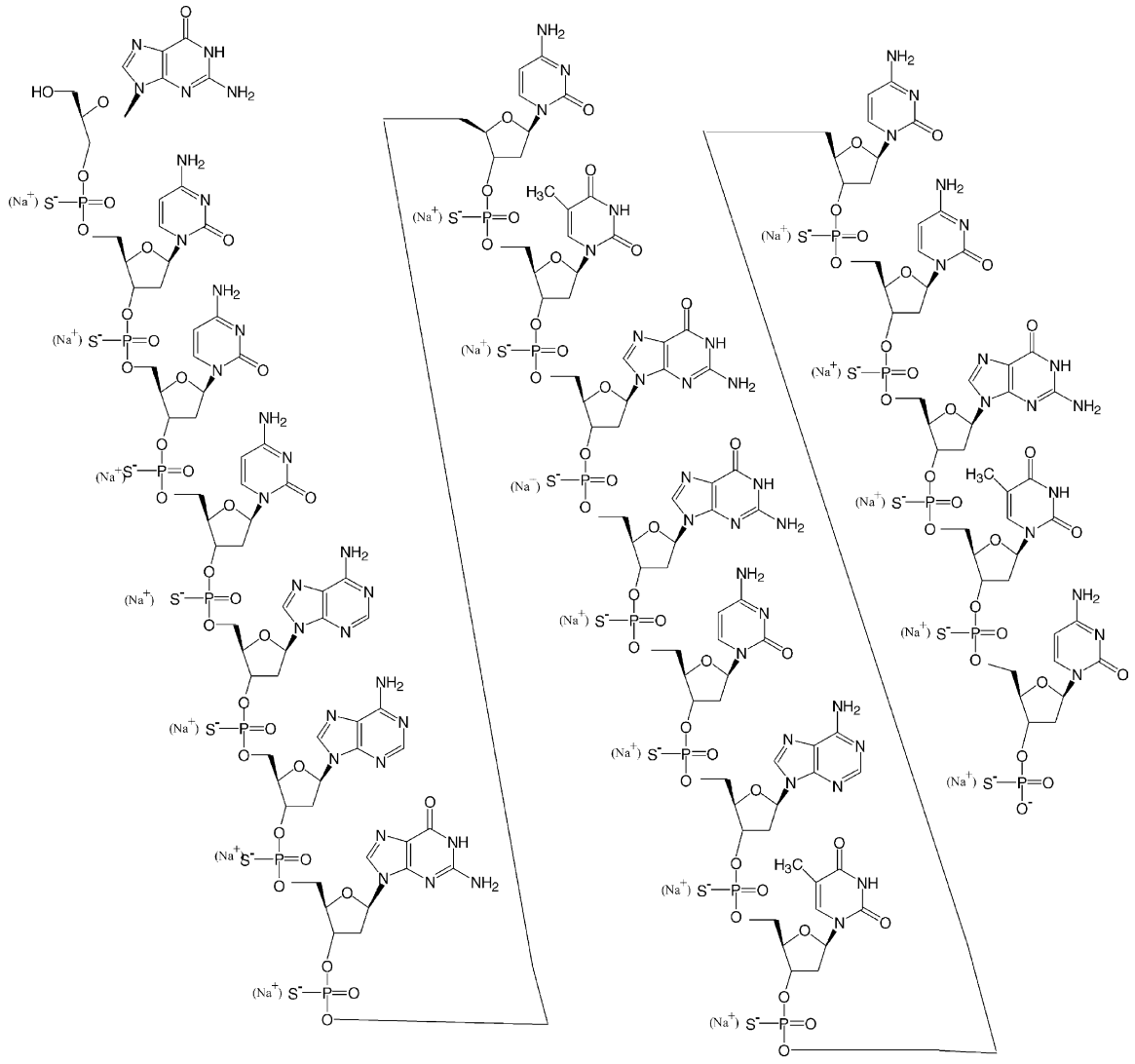


3'-TPT Phosphorothioate Oligonucleotide

a species with a mass of 6438.5 amu, consistent with a 3′-terminal phosphorothioate (3′-TPT) monoester of (n-1)-mer of **1**.

Currently, C_{18} reverse-phase HPLC is used for purification of most phosphorothioate oligonucleotide sequences. This method allows facile separation of 5′-DMT-on oligonucleotide from capped failure sequences. However, since 3′-TPT monoester will also possess a DMT group, RP-HPLC does not discriminate between the species. This is clearly shown in the case of phosphorothioate oligonucleotide **1** (Fig. 1).

Proposed mechanism for formation of 3′-Terminal Phosphorothioate Monoester (3′-TPT)

A reasonable hypothesis is that during the first detritylation step, dA nucleoside attached to solid support undergoes significant depurination leading to an abasic site which on incubation with concentrated aqueous ammonium hydroxide at 55 °C undergoes fragmentation of abasic site, leading to 3′-TPT monoester (Scheme 1). Since this is a single process-related species potentially present at levels of several tenths of one percent, we determined to investigate the species by synthesis and characterization of its biochemical and biological properties. The formation of 3′-TPT is a general phenomenon for oligonucleotides starting with dA at the 3′-end. Recently we demonstrated that considerable depurination of dA attached to solid support occurs leading to the formation of 3′-TPT and this class of process-related species could be eliminated or substantially reduced by a simple reversal of the manner in which the first deoxynucleoside (succinate) is attached to solid support.[2]

Synthesis and Characterization of 3′-TPT Oligonucleotide

Synthesis of oligonucleotide was performed on an Amhersham-Pharmacia Biotech OligoPilot II DNA/RNA synthesizer in a 48-mL stainless column reactor using β-cyanoethyl phosphoramidite synthons (2.0 equiv/coupling, 0.2 M in acetonitrile). 1*H*-tetrazole

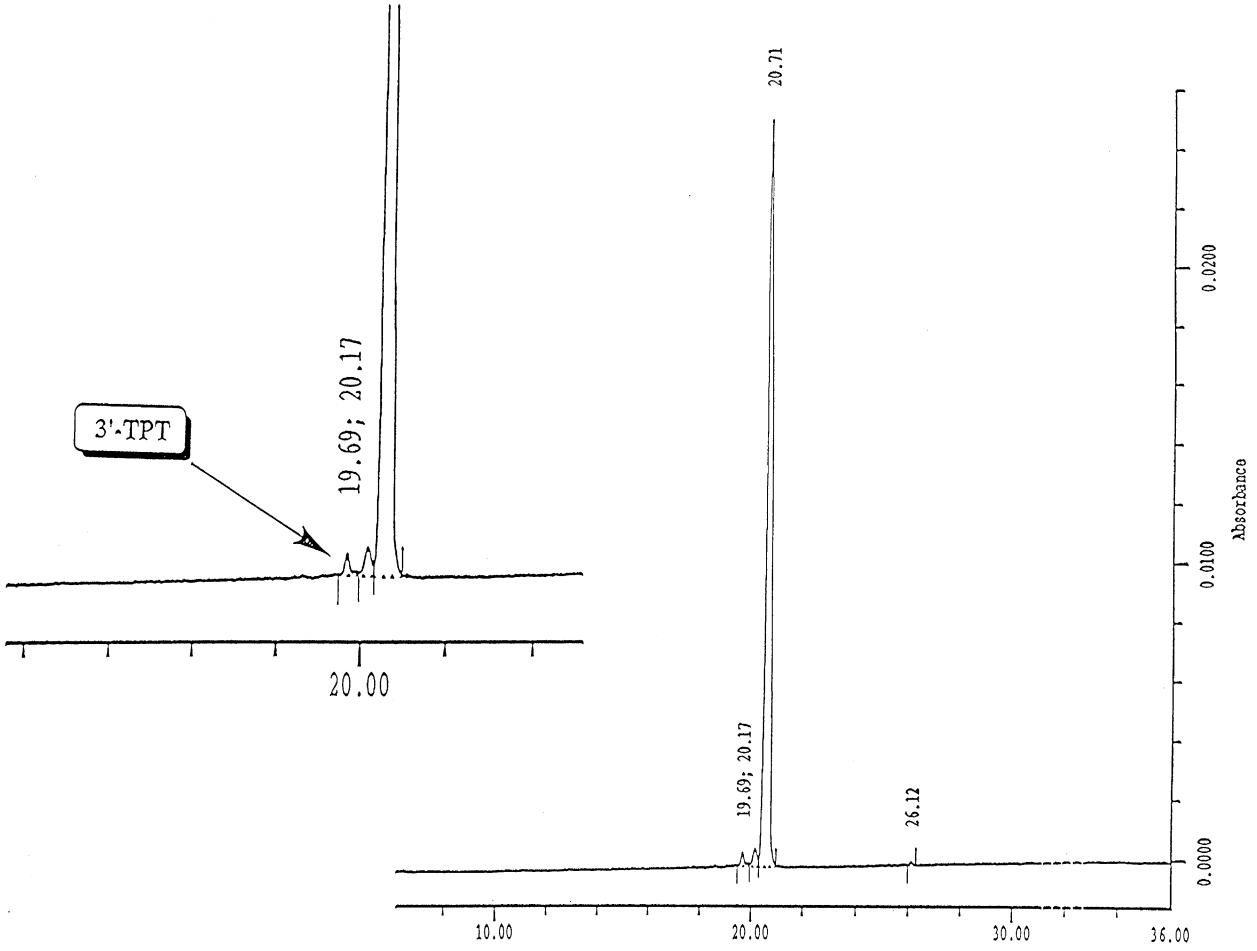
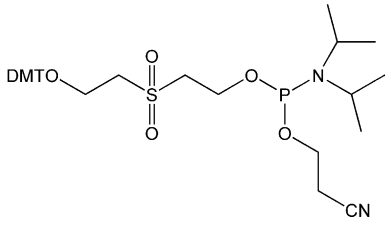
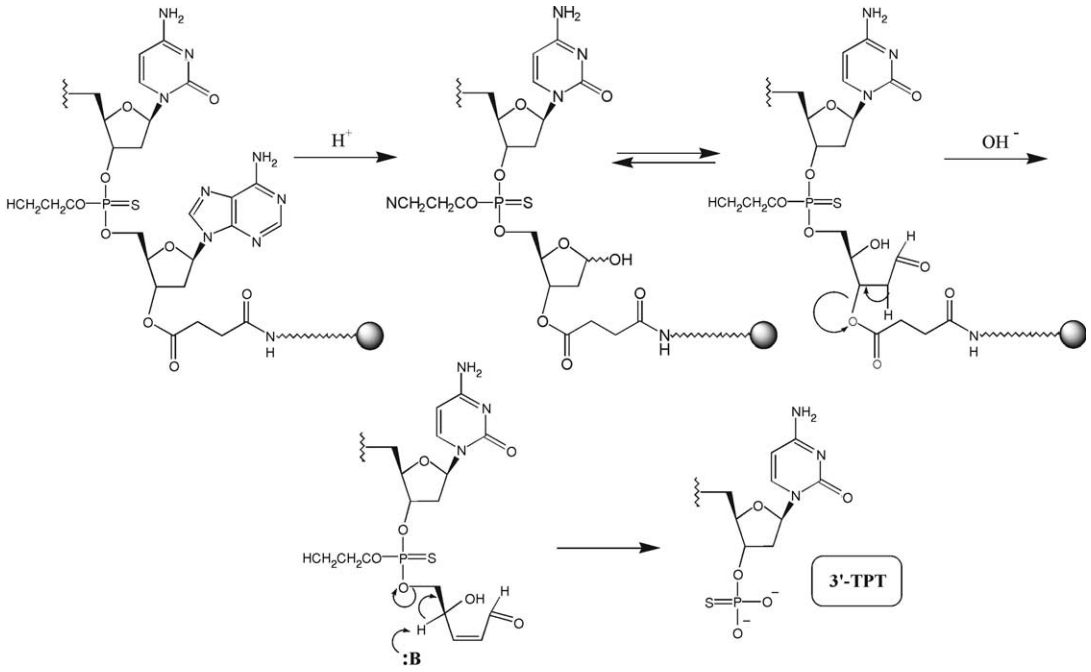


Figure 1. CGE analysis of ISIS 2302 API.

(0.45 M in acetonitrile) was used as activator and phenylacetyl disulfide (PADS) (0.2 M in 3-picoline/CH_3CN, 1:1, v/v) as sulfur transfer reagent.[3,4] Capping reagents were made as recommended by Amersham Biosciences; Cap A: *N*-methylimidazole–CH_3CN (1:4 v/v), Cap B: acetic anhydride–pyridine–CH_3CN (2:3:5, v/v/v). Pharmacia HL 30 Primer support was used with a loading of 94 μmol/g (synthesis scale = 1.4 mmole). 5′-Phosphate-ON reagent was purchased from ChemGenes and used as a 0.2 M solution (2.0 equiv) in CH_3CN. To introduce the 3′-terminal phosphorothioate monoester, commercially available 5′-phosphate-ON reagent was first coupled to Primer T solid support, then the oligonucleotide constructed and final ammonia deprotection gave the desired 3′-TPT functionality. Phosphoramidites, 1*H*-tetrazole and 5′-Phosphate-ON solutions were prepared using anhydrous CH_3CN and were dried further by addition of activated 4 Å molecular sieves. At the end of synthesis, the column containing oligonucleotide was thoroughly dried to determine the yield/μmole (6.72 mg/μmol).[5] The dried 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.[6] The support was filtered, washed with acetonitrile (250 mL) 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, solid filtered using a sintered funnel. The support was washed with water (250 mL), and combined solution concentrated under rotovap. Triethylamine (4 mL) was added and the solution stored in a refrigerator.



The crude DMT-on oligomer was purified using reversed-phase HPLC under standard conditions, fractionated and desired fractions pooled. Detritylation was performed in the usual way, precipitated and lyophilized to afford a colorless amorphous powder (3.8 gm). The purified oligonucleotide was analyzed by CGE (Fig. 2), SAX HPLC (Fig. 3), and by ^{31}P NMR (Fig. 4) spectroscopy.



Scheme 1. Proposed mechanism for formation of 3′-TPT.

Determination of Hybridization Thermodynamics

Experimental

Absorbance versus temperature profiles were measured on a Gilford Response spectrophotometer in a buffer containing 100 mM Na^+, 10 mM phosphate (pH 7.1) and 0.1 mM EDTA. Oligonucleotides and their complements (RNA 10652 and DNA 7822) were combined at 4 μM each strand, heated 5 min at 90 °C and cooled slowly to allow formation of perfect duplexes. Oligonucleotide concentrations were calculated from oligonucleotide absorption at 260 nm at 85 °C and extinction coefficients estimated according to the method of Puglisi and Tinoco.[7] Oligonucleotide solutions were heated at a rate of 0.7 °C/min in 1-cm path length cells and then cooled to confirm reversibility and lack of evaporation. T_m's and free energies of duplex formation were obtained from fits of absorbance versus temperature curves to a two-state model with linear sloping base lines.[8]

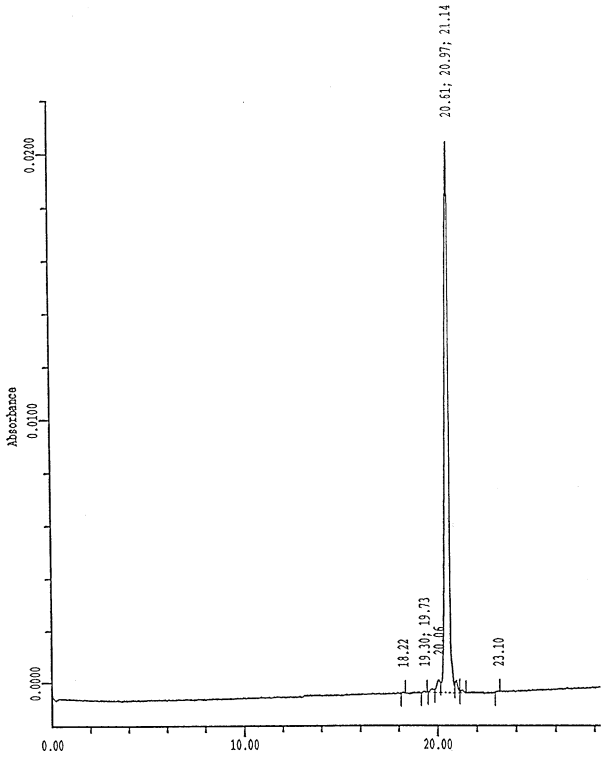


Figure 2. CGE analysis of synthesized 3′-TPT oligonucleotide.

Results and Discussion

Each value of T_m was derived from three experiments and averaged (Fig. 5). Standard deviations are also indicated in Table 1.

In summary, analysis of Table 1 reveals that 3′-TPT does not destabilize the duplex significantly both against DNA and RNA.

RNase H Initial Rate Determination on the Duplex Formed with 3′-TPT

Experimental: ^{32}P labelling of oligoribonucleotides

The oligonucleotide sense strand was 5′-end labeled with ^{32}P using [γ-^{32}P]ATP, T4 polynucleotide kinase, and standard procedures.[9] The labeled RNA was purified by electrophoresis on 12% denaturing PAGE.[10]

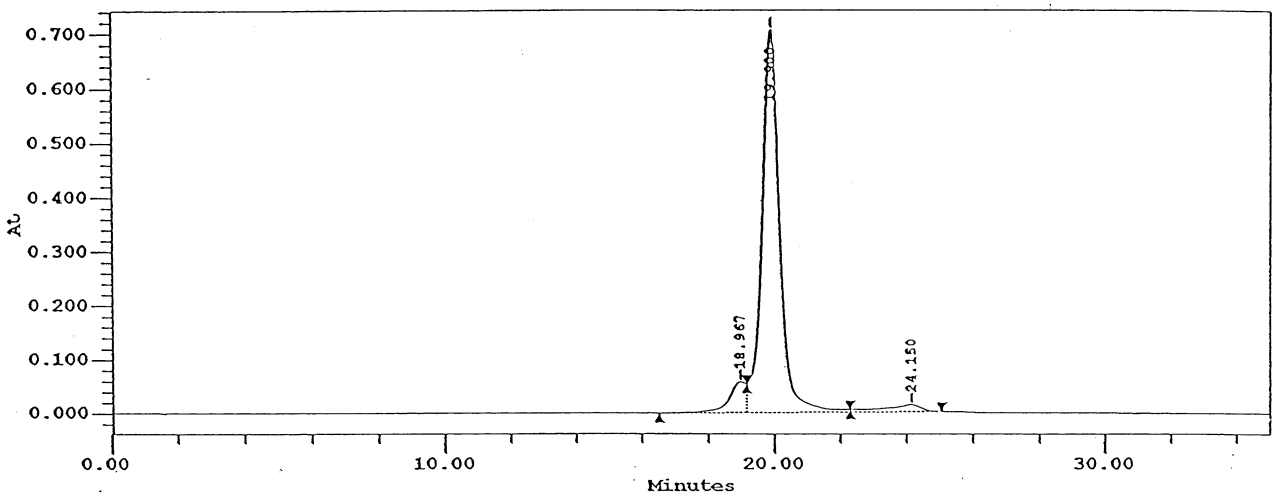


Figure 3. SAX analysis of synthesized 3′-TPT oligonucleotide.

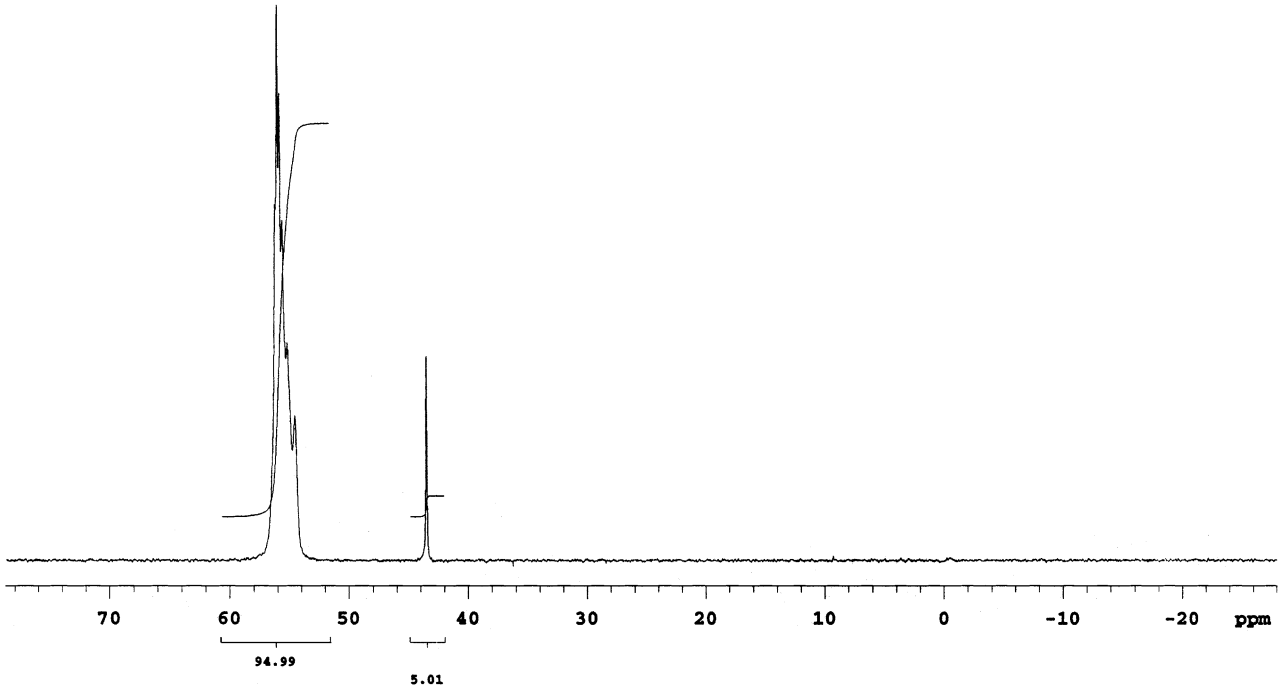


Figure 4. ^{31}P NMR (D_2O) analysis of synthesized 3′-TPT oligonucleotide.

The specific activity of labeled oligonucleotide was approximately 6000 cpm/fmol.

Determination of initial rates

Hybridization reactions were prepared in 120 μL of reaction buffer (20 mM Tris HCl, pH 7.5, 20 mM KCl, 10 mM $MgCl_2$, 0.1 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.[9] Digestion reactions were analyzed at specific time points in 3 M urea and 20 nM EDTA. Samples were analyzed by trichloroacetic acid assay.[11]

Results and Discussion

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

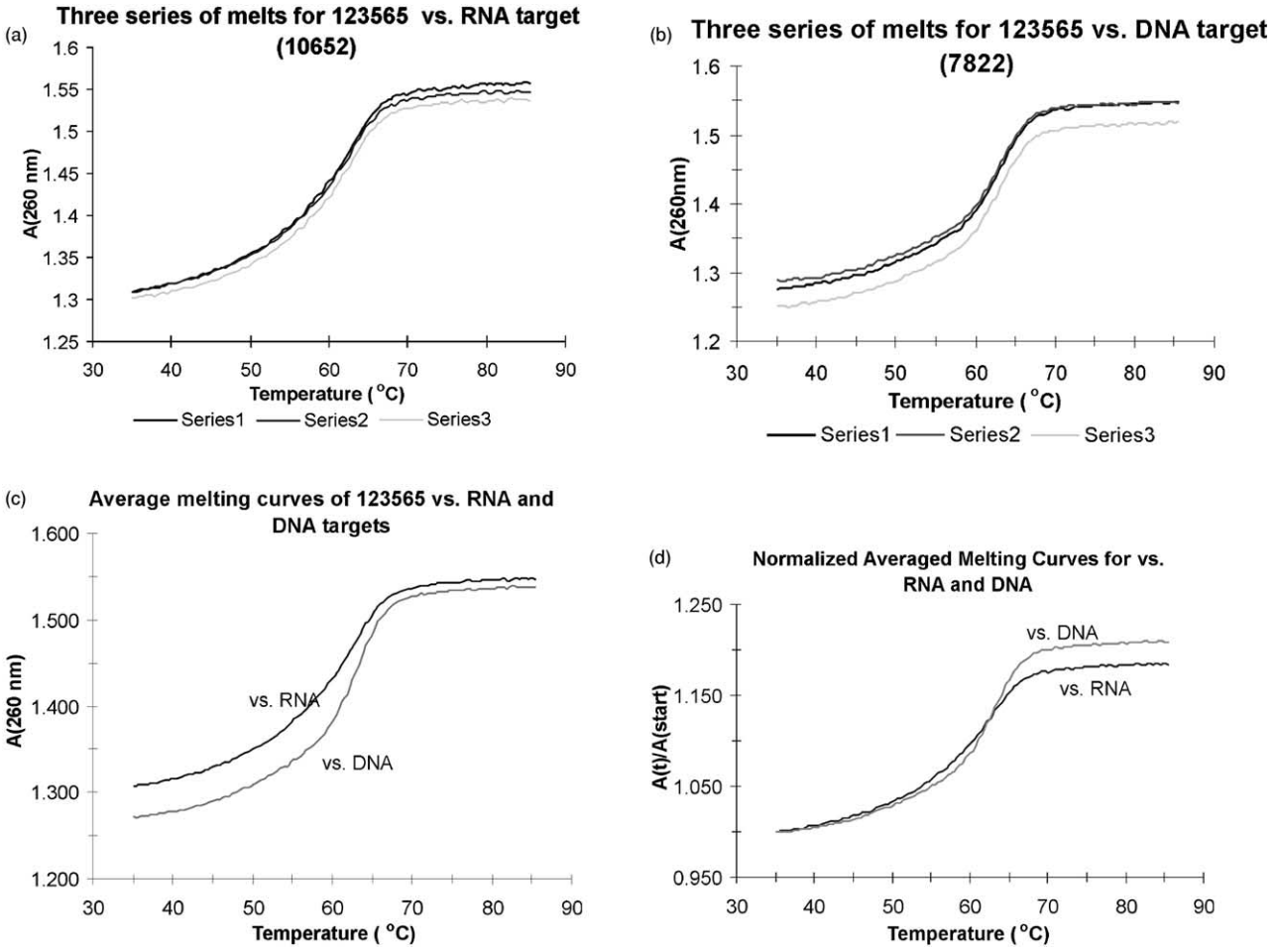


Figure 5. Melting curves.

Table 1.

Analysis	Target	Lot #	T_m	Average T_m	SD
2892	RNA 10652	1	60.95	61.01	0.08
2893	RNA 10652	1	60.97		
2894	RNA 10652	1	61.10		
2892	DNA 7822	7	62.18	62.25	0.06
2893	DNA 7822	7	62.28		
2894	DNA 7822	7	62.28		

Table 2.

Sample	V_o (pM/min)	P	Sequence
2302	0.938±0.018	—	5′-GCCCAAGCTGGCA TCCGTCA
3′-TPT	0.902±0.098	0.001	5′-GCCCAAGCTGGCAT CCGTC-PSO_2

substrate per min) of best-fit line derived from ≥5 data points within the linear portion (<10% of the total reaction) of the plot.[12] The errors reported were based on three trials and is shown (Table 2).

Analysis of (Table 2) shows that the 3′-TPT species behaves similar to the parent drug, namely ISIS 2302.

Conclusion

Synthesis of an authentic sample of 3′-terminal phosphorothioate monoester of 3′-terminal (n-1)-mer of ISIS 2302 has been accomplished. Multiple physical lines of evidence reveal that this process related species is a parent drug-like molecule.

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