INTRODUCTION

The pentadecameric thrombin-binding aptamer1 (TBA, 5′-G1G2T3T4G5G6T7G8T9G10G11T12T13G14G15) has been extensively studied and is well characterized. TBA folds into an intramolecular antiparallel G-quadruplex that consists of two G-quartets that are connected by two TT loops and a central TGT loop in a chair-like conformation. TBA is reported to interact with its target, thrombin, mainly through its loop TGT loop in a chair-like conformation. TBA is reported to G-quartets that are connected by two TT loops and a central intramolecular antiparallel G-quadruplex that consists of two regions, particularly the TT loops.2 Owing to its promising nature, could help to ease the strain in the loop regions of TBA and had a maximum stabilizing effect in the T7 position formed a quadruplex with the highest thermal stability. It also resulted in enhanced anticoagulant activity that allowed a one-third reduction in the dose, relative to TBA. Further, TBA-7T exhibited enhanced nuclease resistance properties to both endo- and exonucleases.

Methoxymethyl Threofuranosyl Thymidine (4′-MOM-TNA-T) at the T7 Position of the Thrombin-Binding Aptamer Boosts Anticoagulation Activity, Thermal Stability, and Nuclease Resistance

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ABSTRACT: The synthesis of 4′-methoxymethyl threofuranosyl (4′-MOM-TNA) thymidine and derived oligomers of the G-rich thrombin-binding aptamer (TBA) sequence is reported. The G-quadruplex stability, anticoagulant activity, and the enzymatic stability of these oligomers bearing the 2′-3′-phosphodiester backbone as single substitutions in the loop regions are studied. Amongst all the oligomers, TBA-7T bearing the 4′-MOM-TNA unit at the T7 position formed a quadruplex with the highest thermal stability. It also resulted in enhanced anticoagulant activity that allowed a one-third reduction in the dose, relative to TBA. Further, TBA-7T exhibited enhanced nuclease resistance properties to both endo- and exonucleases.
Inclusion of a methoxy substituent in the ribose/deoxyribose sugar moiety in nucleosides is known to impart stability against nuclease degradation. Methoxy substitutions at the 2′-position and 4′-position have been reported to render the oligonucleotides resistant to exonuclease and endonucleases, depending on the position of the methoxy-substituted nucleotide in the oligomer. The -OMe substitution was reported to additionally increase the stability of the resulting duplexes with complementary DNA/RNA. 16,18 5′-OMe substitutions in siRNA duplexes were reported to be useful in controlling the targeting specificity in RNAi. 19 We recently showed that inclusion of a single 2′-OMe-substituted nucleoside in the loop region of TBA was well tolerated in spite of the sugar being in the N-type puckered conformation. 20

The methoxymethyl substituent at C4′ that is reported in this work may be expected to contribute toward enhancing the nuclease resistance properties of the resulting oligomers, besides favorably influencing hydration in its vicinity. Thus, in this paper, we report the synthesis of 4′-MOM-TNA and study the effect of single nucleotide substitution in the loop regions of TBA on the stability of the G-quadruplex and its thrombin-binding, anticoagulant, and endo- and exonuclease resistance properties.

### RESULTS AND DISCUSSION

#### Synthesis of 4′-Methoxymethyl TNA-T Monomers

A straightforward route for the synthesis of 4′-MOM-TNA-T phosphoramidite was followed using commercially available d-xylose as the starting material (Scheme 1). D-Xylose 1 was transformed to 1,2-O-isopropylidene-α-D-xylofuranose 2 using acetonide, conc. H2SO4 and then Na2CO3 in a one-pot reaction. 21 Monomethylation of the primary hydroxyl group by methyl iodide in the presence of silver oxide yielded 3. The secondary hydroxyl group was converted to its allyloxycarbonyl derivative 4 in 70% yield. The compound 4 was converted into its diacetate 5 after acetonide removal in the presence of AcOH and Ac2O and a catalytic amount of H2SO4. Compound 5 on treatment with N,O-bis(trimethylsilyl)acetamide (BSA), thymine, and TMS-OTf yielded compound 6 exclusively as the β-anomer. The alcohols group was selectively cleaved using Pd(0) to get 7, and the 3′-hydroxyl group was converted to its DMT derivative using 2,4,6-collidine as a base to get 8. The 2′-hydroxyl group in compound 8 was deprotected by ammonolysis to obtain compound 9, which was phosphitylated using 2-cyanoethyl-N,N-disopropylchlorophosphine to yield the phosphoramidite monomer 10. All compounds were characterized by 1H and 13C NMR and HRMS analysis, and the phosphoramidite 10 was characterized by 31P NMR spectroscopy (Supporting Information, Figures S1 and S2).

#### Synthesis of TBA Oligomers

The synthesis of TBA oligomers was carried out on the solid phase using phosphoramidite chemistry on a Bioautomation MM4 DNA synthesizer. An extended coupling time of 2 min was used for the 4′-MOM-TNA-T units, which were introduced as single substitutions at the 3′-, 4′-, 7′-, 9′-, 12′-, or 13′-position in the loop regions of TBA to yield TBA oligomers TBA-3T, TBA-4T, TBA-7T, TBA-9T, TBA-12T, and TBA-13T respectively. For comparison, 3′-deoxy-thymidine-2′-phosphoramidite was used to introduce a single 2′-5′-linked T unit at the seventh or ninth position of TBA (TBA-7T, TBA-9T respectively). This isodNA unit presents the sugar in the N-type pucker with an extended backbone. 22 All the synthesized oligomers are listed in Table 1 were purified by HPLC, their identity was confirmed by MALDI-TOF mass analysis, and their purity was reconfirmed by analytical HPLC and gel electrophoresis (Supporting Information, Figures S3 and S4).

#### Evaluating G-Quadruplex Formation and Thermal Stability

CD spectra were recorded to evaluate the influence of the 4′-MOM-TNA-T and 2′-5′- backbone on the overall G-quadruplex structure of TBA. As reported for TBA, all the

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**Scheme 1. Synthesis of 4′-MOM-TNA-T Phosphoramidite**

![Scheme 1](image-url)

**Reagents and conditions:** (i) acetone, conc. H2SO4, 30 min, Na2CO3, 2.5 h, 89%; (ii) MeI, Ag2O, dry CH3CN, 64%; (iii) Alloc-Cl, dry pyridine, dry CH2Cl2, room temperature (rt), 3 h, 70%; (iv) AcOH,Ac2O/H2SO4 (10:1:0.1), rt, overnight, 74%; (v) thymine, CH3CN, BSA, 70 °C and then TMS-OTf, 0 °C and then reflux, 3 h, 65%; (vi) PPh3, Pd(dba)2, piperidine, CH2Cl2, rt, 15 min, 65%; (vii) DMT-Cl, 2,4,6-collidine, CH2Cl2, rt, 24 h, 76%; (viii) MeOH, aq. ammonia, 87%; (ix) 2-cyanoethyl-N,N-diisopropylchlorophosphine, DIPEA, dry CH2Cl2, rt, 1 h, 60%.
modified oligomers displayed CD signatures typical of antiparallel G-quadruplexes \(^\text{25}\) in the presence of K\(^+\) ions, with maxima near 290 nm and minima near 260 nm (Figure 2). The differences in the amplitude of the CD signal could reflect the efficiency of base stacking. TBA oligomers containing the 4′-MOM-TNA unit at the T7 position was particularly different from the rest of the oligomers and showed a stronger CD signal than even TBA. Further, TBA-7T also displayed the characteristic bands of the antiparallel G-quadruplexes with the highest thermal stability. This indicates that, although 4′-MOM-TNA has the sugar in the N-type of pucker, together with a shorter backbone, it is well accommodated at the T7 position in the TGT loop of TBA. A similar stabilization of \(\sim 10\) °C for single substitutions was reported when the T4 or T13 units were replaced by S-fluoro-2′-deoxyuridine, where the stabilization was found to be largely enthalpy-driven. The changes in enthalpy and entropy for the two-state transition observed for the TBA oligomers were calculated from a van’t Hoff analysis of the CD equilibrium melting curves.\(^{23,24}\) The data are presented in Table 3, and stable quadruplex in the presence of Na\(^+\) ions (Supporting Information, Figures S5b and S6b) with \(T_m = 51\) °C (\(\Delta T_m = +29\) °C in comparison to TBA) and also in the absence of any added cations (Supporting Information, Figure S7 and Table S1; \(T_m = 28\) °C, \(\Delta T_m = +9\) °C in comparison to TBA). Thus, among all the oligomers studied, TBA-7T was found to form quadruplexes with the highest thermal stability. This indicates that, although 4′-MOM-TNA has the sugar in the N-type of pucker, together with a shorter backbone, it is well accommodated at the T7 position in the TGT loop of TBA. A similar stabilization of \(\sim 10\) °C for single substitutions was reported when the T4 or T13 units were replaced by S-fluoro-2′-deoxyuridine, where the stabilization was found to be largely enthalpy-driven. The changes in enthalpy and entropy for the two-state transition observed for the TBA oligomers were calculated from a van’t Hoff analysis of the CD equilibrium melting curves.\(^{23,24}\) The data are presented in Table 3, and

Table 1. TBA Oligomers of the Study

| entry no. | code | sequencea | S’→3’ | MALDI-TOF mass (Da) calcld./obsd. |
|-----------|------|-----------|--------|----------------------------------|
| 1         | TBA  | ggttggggttgg  |        | 4726/4730                         |
| 2         | TBA-3T | ggtT\text{MOM-TNA}ggttggggttgg  |        | 4756/4750                         |
| 3         | TBA-4T | ggtT\text{MOM-TNA}ggttggggttgg  |        | 4756/4753                         |
| 4         | TBA-7T | ggtT\text{MOM-TNA}ggttggggttgg  |        | 4756/4754                         |
| 5         | TBA-9T | ggttggT\text{MOM-TNA}ggttgg  |        | 4756/4754                         |
| 6         | TBA-12T | ggttggT\text{MOM-TNA}ggttgg  |        | 4756/4752                         |
| 7         | TBA-13T | ggttggT\text{MOM-TNA}ggttgg  |        | 4756/4753                         |
| 8         | TBA-7Tiso | ggtgT\text{MOM-TNA}ggttgg  |        | 4726/4729                         |
| 9         | TBA-9Tiso | ggtgT\text{MOM-TNA}ggttgg  |        | 4726/4731                         |

\(\text{a}\)The lower case letters indicate unmodified DNA, and upper case letters indicate the modified units. \(\text{T\text{MOM-TNA}}\) indicates 4′-MOM-TNA-T, while \(\text{T\text{MOM-TNA}}\) indicates 2′-S-linked 3′-deoxy-thymidine.

Figure 2. CD spectra of the TBA oligomers in the presence of K\(^+\) ions. Spectra were recorded in 10 mM potassium phosphate buffer, pH 7.5, containing 100 mM KCl at 4 °C.

Table 2. CD Melting Temperatures of the TBA Oligomers \(^a\)

| oligomer | \(T_m\) °C (K\(^+\)) \(\Delta T_m\) °C | \(T_m\) °C (Na\(^+\)) | \(T_m\) °C (thrombin) |
|----------|---------------------------------|-----------------|-----------------|
| TBA      | 50                              | 22              | 20              |
| TBA-3T   | 40 (−10)                        | 13 (−9)         | nd              |
| TBA-4T   | 37 (−13)                        | 17 (−5)         | nd              |
| TBA-7T   | 60 (+10)                        | 51 (+29)        | 32 (+12)        |
| TBA-9T   | 34 (−16)                        | nd              | nd              |
| TBA-12T  | 47 (−3)                         | 22 (0)          | nd              |
| TBA-13T  | 34 (−16)                        | 19 (−3)         | nd              |
| TBA-7Tiso| 47 (−3)                         | nd              | nd              |
| TBA-9Tiso| 37 (−13)                        | nd              | nd              |

\(\text{a}\)Values in parentheses indicate the difference in \(T_m\) (\(\Delta T_m\)) relative to TBA. nd = not determined. \(\text{b}\)Experiments were performed with a strand concentration of 5 \(\mu\)M in 10 mM potassium phosphate buffer (pH 7.5) containing 100 mM KCl. \(\text{c}\)Experiments were performed with a strand concentration of 5 \(\mu\)M in 10 mM sodium phosphate buffer (pH 7.5) containing 100 mM NaCl. \(\text{d}\)Experiments were performed with a strand concentration of 5 \(\mu\)M in water containing 5 NIH units of thrombin per mL. and were repeated at least thrice. The values are accurate to \(±1\) °C.

Table 3. Thermodynamic Parameters Obtained from the CD Melting Plots by van’t Hoff Analysis

| oligomer    | \(\Delta H\) (kJ mol\(^{-1}\)) | \(\Delta S\) (kJ mol\(^{-1}\) K\(^{-1}\)) | \(\Delta H\) (kJ mol\(^{-1}\)) | \(\Delta S\) (kJ mol\(^{-1}\) K\(^{-1}\)) |
|-------------|-------------------------------|---------------------------------|-------------------------------|---------------------------------|
| TBA         | −116                          | −0.36                           | −92                           | −0.31                           |
| TBA-3T      | −119                          | −0.36                           | −95                           | −0.34                           |
| TBA-4T      | −79                           | −0.26                           | −105                          | −0.34                           |
| TBA-7T      | −125                          | −0.38                           | −105                          | −0.34                           |
| TBA-9T      | −110                          | −0.36                           | −105                          | −0.34                           |
| TBA-12T     | −80                           | −0.25                           | −105                          | −0.34                           |
| TBA-13T     | −92                           | −0.29                           | −105                          | −0.34                           |

representative plots of \(\ln K\) versus \(1/T\) are shown in the Supporting Information (Figure S8). The data suggest that the stabilization observed with TBA-T7 is largely enthalpy-driven. The effect of the 4′-MOM-TNA unit on the G-quadruplex and its thermal stability may be explained in context of the neighboring nucleosides, together with the compact nature of the 2′-3′-backbone with the sugar in an N-type conformation. We previously reported\(^{20}\) that N-type sugar modifications in

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the form of 2′-OMe-substituted nucleosides imparted controlled compactness and were favorably accommodated as single substitutions in the loop regions of TBA. G10 is a part of the G-tetrad upon which the T9 thymine is reported to stack flatly, contributing to the thermal stability of the quadruplex and the intensity of the observed CD spectrum. Replacement of T9 by a 4′-MOM-TNA unit would directly impact this interaction, the shorter backbone together with the N-type of sugar pucker probably leading to excessive compactness in this case, and resulting in destabilization of the quadruplex. Replacement of T4 or T13, which are also reported to be stacked on the G-tetrad, by 4′-MOM-TNA, therefore similarly result in a large destabilization of the quadruplex. On the other hand, the T rings of T3, T12, and T7 are tilted away from their succeeding nucleobase, and replacement of these causes a lower degree of destabilization. T7 is positioned sideways into the wide groove of the quadruplex, and its replacement by 4′-MOM-TNA probably leads to compactness that is favorable in this case, which also possibly enhances the stacking of G8 on the G-tetrad, leading to an increased intensity of the observed CD maximum and a concomitant increased thermal stability of the quadruplex.

NMR studies were carried out with the most stable G-quadruplex, that is, of TBA-7T. In the 1H NMR spectrum, the characteristic hydrogen-bonded imino proton resonances of a G-quadruplex were observed between 11.5 and 12.5 ppm in the presence of K+ ions (Figure 3a). These signals appeared slightly downfield to those observed for TBA, probably indicative of the stronger hydrogen-bonding in the case of TBA-7T. A similar downfield shift in the case of stronger hydrogen-bonding has been reported for phenolic compounds. The signal near 11.2 ppm observed for TBA was shown to correspond to the imino proton signal of T7, which is close to the imino proton resonances of T4 and T13, all of which are not involved in the H-bonded G-tetrad. These signals at 11.2 ppm were not apparent for TBA-7T or are possibly also shifted slightly, displaying a difference in comparison to TBA. The imino proton resonances characteristic of H-bonded G-tetrads in quadruplexes were observed even in water, with no added cation (Figure 3b), indicating the capability of the oligomers to fold into stable quadruplexes in cation-free conditions.

**Antithrombin Activity Studies.** We further investigated whether the higher thermal stability of the TBA-7T G-quadruplex would translate into its antithrombin activity, although it has been reported that the two may not necessarily be related. TBA-7T was found to form a stable complex with thrombin, with a Tm of 32 °C (Table 2 and Supporting Information, Figure S9). The antithrombin activity of the TBA oligomers was investigated by measuring the percent transmittance at 450 nm as a function of time (Supporting Information, Figure S10) and recording the induction time for conversion of fibrinogen to fibrin in the presence of thrombin. TBA was found to slow down the coagulation, evident from an increased induction time in comparison to that in the absence of any added oligomer as reported previously (Figure 4a).

The induction time for TBA-7T (26.7 min) was higher than for TBA (11.2 min) at the same concentration, thus offering the possibility to reduce the concentration of TBA oligomer required to bring it within a more acceptable therapeutic range. Toward further exploring this possibility, we carried out the clotting assay at successively decreasing concentrations of TBA-7T and found that TBA-7T showed a comparable induction time to TBA at less than one-third of its concentration (Figure 4b). These experiments thus provide conclusive evidence that TBA-7T is capable of forming a stable G-quadruplex with similar structural topology as TBA and also positively impacting the assigned function of TBA. In comparison, TBA-7Tiso, with an induction time of 7.2 min, was less efficient than even TBA at inhibiting clotting (Figure 4a). Anticoagulation experiments performed with human plasma and thrombin further demonstrated TBA-7T to be as good as TBA (Figure S11).

**Nuclease Stability Studies.** In addition to enhancing the thermal stability and anticoagulation ability of TBA, another objective of introducing modified units into its sequence was to increase the stability of the resulting oligomers toward degradation by nuclease. The stability of TBA and TBA-7T oligomers to nuclease degradation was therefore compared. Snake venom phosphodiesterase (SVPD), which possesses predominantly 3′-exonuclease activity, and S1 nuclease, an endonuclease, were used. We reasoned that any hydrolysis of

![Figure 3](image-url)

**Figure 3.** G-quadruplex imino proton region in the 1H NMR spectra of TBA-7T and TBA at a concentration of 150 μM at 4 °C (a) in the presence of 100 mM K+ and (b) in its absence.

![Figure 4](image-url)

**Figure 4.** Antithrombin activity obtained from transmittance measurements at 450 nm and plotted as induction time (a) in the presence of selected TBA oligomers of the study and (b) for TBA-7T at decreasing concentrations in comparison to TBA. Control represents the measurement with fibrinogen (Sigma, product no. F3897) and thrombin (bovine thrombin, Tulip Diagnostics (P) Ltd.) in the absence of any added TBA oligomer.
phosphodiester linkages would result in a loss of the quadruplex structure, which would be immediately apparent from the CD spectra of the TBA oligomer. The percent change in the CD amplitude at 295 nm, characteristic of the G-quadruplex, was therefore monitored as a function of time to obtain a measure of the nuclease stability of the oligomer. TBA-7T was found to be much stable (t1/2 > 120 min) than TBA, which exhibited a half-life of ∼35 min when treated with SVPD (Figure 5a). A similar trend was observed for the TBA, which exhibited a half-life of ∼35 min when treated with SVPD (Figure 5a). A similar trend was observed for the oligomers with S1 nuclease (Figure 5b), where TBA displayed a t1/2 of ∼35 min, while for TBA-7T, this was >105 min. In the absence of nuclease, the change in the CD amplitude at 295 nm was very minimal (Supporting Information, Figure S12). This was further confirmed when the reaction was monitored by HPLC analysis (Supporting Information, Figures S13 and S14).

CONCLUSIONS

In conclusion, the design and synthesis of 4′-MOM-TNA is reported for the first time. Its effect when substituted at the T7 position of TBA was found to be beneficial, causing a large increase in thermal stability that also translated into anticoagulating activity. The stabilizing effect was even better than observed earlier with UNA, indicating the optimum attributes offered by the 4′-MOM-TNA unit when placed at the T7 position. Further, TBA-7T was found to have enhanced stability toward endo- and exonucleases. Its strong anticoagulating activity allowed for a 3-fold reduction in the dose required toward potential therapeutic application.

EXPERIMENTAL SECTION

General Remarks. CH3CN and CH3Cl2 were dried over CaH2 and stored over 4 Å molecular sieves, while pyridine and Et3N were dried over KOH and stored over KOH. The progress of reactions was monitored by TLC (pre-coated silica gel GF254 sheets (Merck 5554)). TLCs were run in petroleum ether/EtOAc or CH2Cl2/MeOH systems and were visualized with UV light and iodine spray and/or by spraying perchloric acid solution and heating. Column chromatographic separations were performed using silica gel 60–120 mesh (Merck) or 200–400 mesh (Merck).

1H and 13C NMR spectra were recorded on a Bruker AC-200, AC-400, or AC-500 NMR spectrometer. The chemical shifts are referred to internal TMS for 1H and chloroform-d for 13C NMR. Mass spectra were recorded on a Q Exactive Hybrid Quadrupole Orbitrap Mass spectrometer (Thermo Fisher Scientific), MALDI-TOF spectra were recorded on a SCIEX TOF/TOF 5800 system, and the matrix used for analysis was THAP (2,4,6-trihydroxyacetophenone)/ammonium citrate (2:1). UV experiments were carried out on a Varian Cary 300 UV-Visible spectrophotometer equipped with a Peltier-controlled cell holder. CD spectra were recorded on a Jasco J-815 spectropolarimeter equipped with a Peltier-controlled cell holder.

Experimental Procedures and Spectral Data. 1,2-O-Isopropylidene-α-L-xylufuranose (2). Compound 2 was synthesized by a reported procedure.23 Accordingly, finely powdered D-xylene 1 (10.00 g, 67.1 mmol) was dissolved in aceton (260 mL) containing H2SO4 (10.0 mL, 96%, 66.0 mmol) and stirred for 30 min. A solution of Na2CO3 (13.00 g, 122.65 mmol) in H2O (112 mL) was carefully added while cooling to maintain the temperature at 20 °C. After stirring for a further 2.5 h, solid Na2CO3 (7.00 g, 66.0 mmol) was added to bring the pH to 7.0. Na2SO4 was filtered off and washed with aceton. The combined filtrates were evaporated to yield crude 2 that was purified by filtration through silica gel using 30:1 CH2Cl2/MeOH, and pure 2 was obtained as a syrup, which crystallized on standing. Yield 11.3 g, 89%. 1H NMR (400 MHz, CDCl3): δ 1.23 (s, 3H), 1.40 (s, 3H), 2.08 (br, 2H), 4.08–4.11 (m, 2H), 4.39 (br, 1H), 4.44 (d, J = 3.5 Hz, 1H), 4.63 (d, J = 4.3 Hz, 1H), 5.88 (d, J = 3.5 Hz, 1H) ppm; 13C NMR (50 MHz, CDCl3): δ 25.9, 26.8, 60.2, 75.5, 79.4, 85.2, 104.5, 111.5 ppm. HRMS: mass calculated for C9H16O5Na (M + Na)+ 213.0734, observed (M + Na)+ 213.0734.

5-O-Methyl-1,2-O-isopropylidene-α-L-xylufuranose (3). To a solution of 2 (1.00 g, 5.2 mmol) and CH3I (0.46 mL, 7.8 mmol) in 20 mL of acetonitrile, Ag2O (1.40 g, 6.2 mmol) was added followed by vigorous stirring of the reaction mixture at room temperature for 12 h. It was then filtered, and the filtrate was concentrated under vacuum. Purification by column chromatography (product eluted in 50% EtOAc in petroleum ether) gave 3 (0.68 g) as a white solid in 64% yield. 1H NMR (200 MHz, CDCl3): δ: 1.32 (s, 3H), 1.49 (s, 3H), 3.44 (s, 3H), 3.80–3.84 (m, 1H), 3.89 (t, J = 3.6 Hz, 2H), 4.19–4.24 (m, 1H), 4.29 (t, J = 2.8 Hz, 1H), 4.52 (d, J = 3.7 Hz, 1H), 5.98 (d, J = 3.8 Hz, 1H) ppm; 13C NMR (50 MHz, CDCl3): δ: 26.1, 26.7, 30.9, 71.0, 76.4, 76.6, 85.4, 104.8, 111.6 ppm; HRMS: mass calculated for C7H12O3Na (M + Na)+ 227.0890, observed (M + Na)+ 227.0890.

5-O-Methyl-3-allyloxy 1,2-O-isopropylidene-α-L-xylufuranose (4). To an ice-cooled solution of compound 3 (0.80 g, 3.9 mmol) in dry dichloromethane (10 mL) and anhydrous pyridine (0.75 mL), allyloxycarbonyl chloride (0.47 mL, 4.7 mmol) was added dropwise followed by stirring at room temperature for 3 h when TLC indicated consumption of the

Figure 5. Quadruplex stability of TBA and TBA-7T toward (a) SVPD (6.6 U/μL) and (b) S1 nuclease (53.4 U/mL).
starting material. Water was added, and the reaction mixture was extracted with CH$_2$Cl$_2$ followed by drying of the organic extracts over sodium sulfate. The crude sticky compound 4 was purified by column chromatography (eluted in 25% EtOAc in petroleum ether) to give pure 4 (0.64 g) as a colorless thick liquid in 70% yield. $^1$H NMR (200 MHz, CDCl$_3$): $\delta$: 1.31 (s, 3H), 1.51 (s, 3H), 3.38 (s, 3H), 3.61 (d, $J = 1.3$ Hz, 1H), 3.64 (s, 1H), 4.44 (td, $J = 5.8$, 3.0 Hz, 1H), 4.59 (d, $J = 3.8$ Hz, 1H), 4.65 (dt, $J = 5.8$, 1.3 Hz, 2H), 5.11 (d, $J = 3.0$ Hz, 1H), 5.26–5.44 (m, 2H), 5.81–6.06 (m, 2H) ppm; $^{13}$C NMR (50 MHz, CDCl$_3$): $\delta$: 26.2, 26.6, 59.3, 68.9, 69.5, 77.7, 79.8, 83.2, 104.7, 112.2, 119.4, 131.1, 153.9 ppm. HRMS: mass calculated for C$_{13}$H$_{19}$O$_7$N$_2$ (M + H)$^+$ 311.1101, observed (M + Na)$^+$ 311.1101.

5′-O-Methyl-3′-allyloxy 1,2-di-O-acetyl-D-xylofuranose (5). Compound 4 was subjected to acetonide deprotection and conversion to its 1,2-di-O-acetyl derivative according to the procedure described earlier for a similar derivative. Thus, the desacipated compound 4 (1.20 g, 3.6 mmol) was dissolved in anhydrous CH$_2$Cl$_2$, and the organic layer was washed by water and dried over sodium sulfate. The crude sticky compound 4 was purified by column chromatography using petroleum ether and ethyl acetate as eluants. Yield: 0.51 g, 65%. $^1$H NMR (200 MHz, CDCl$_3$): $\delta$: 1.93 (d, $J = 1.1$ Hz, 3H), 2.13 (s, 3H), 3.47 (s, 3H), 3.84–3.87 (m, 2H), 4.16–4.32 (m, 2H), 5.13–5.15 (m, 1H), 5.74 (d, $J = 2.5$ Hz, 1H), 7.46 (d, $J = 1.2$ Hz, 1H), 8.81 (br, 1H) ppm; $^{13}$C NMR (50 MHz, CDCl$_3$): $\delta$: 12.4, 20.6, 59.3, 70.3, 74.3, 80.4, 82.1, 89.8, 110.8, 137.5, 150.4, 164.2, 170.0 ppm. HRMS: mass calculated for C$_{13}$H$_{19}$O$_7$N$_2$ (M + H)$^+$ 315.1184, observed (M + H)$^+$ 315.1187.

5′-O-Methyl-3′-dimethoxytrityl-2′-O-acetyl thymidine (8). To a solution of compound 7 (0.50 g, 1.6 mmol) in anhydrous CH$_2$Cl$_2$ (10 mL), 4,4′-dimethoxytritylchloride (1.02 g, 3 mmol) and 2,4,6-collidine (1.3 mL, 9 mmol) were added. Upon stirring the reaction mixture for 24 h at room temperature, TLC examination revealed the appearance of the product and disappearance of the starting material. The reaction was quenched by addition of MeOH (1 mL), diluted with CH$_2$Cl$_2$ (50 mL), and washed with NaHCO$_3$ and water. The organic layer was dried over sodium sulfate, and solvents were removed to afford the crude product that was purified by column chromatography on silica gel (pre-neutralized with Et$_3$N). Compound 8 eluted in MeOH (2%) in CH$_2$Cl$_2$. Yield: 0.74 g, 76%. $^1$H NMR (500 MHz, CDCl$_3$): $\delta$: 0.94 (d, $J = 0.9$ Hz, 3H), 1.90 (s, 3H), 3.43 (s, 3H), 3.51 (s, 1H), 3.47–3.53 (m, 2H), 3.71 (s, 3H), 3.72 (s, 3H), 4.38 (t, $J = 5.0$ Hz, 1H), 4.64 (t, $J = 5.0$ Hz, 1H), 5.71 (d, $J = 4.6$ Hz, 1H), 6.76 (dd, $J = 2.3$, 9.2 Hz 4H), 7.17–7.24 (m, 2H), 7.28 (s, 2H), 7.37–7.40 (m, 3H), 7.69 (d, $J = 1.4$ Hz, 1H) ppm; $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$: 12.4, 20.5, 55.2, 58.9, 70.8, 74.9, 78.5, 79.0, 85.6, 87.5, 110.9, 113.3, 127.3, 127.9, 128.0, 128.4, 128.5, 130.2, 130.4, 132.0, 132.1, 135.3, 135.4, 136.6, 144.6, 150.4, 158.9, 159.0, 163.8, 169.5 ppm. HRMS: mass calculated for DMT tr cleaved fragment C$_{14}$H$_{21}$O$_7$Na (M + Na)$^+$ 337.1006, observed (M + Na)$^+$ 337.1006.

5′-O-Methyl-3′-dimethoxytrityl-2′-hydroxy thymidine (9). To a solution of compound 8 (0.70 g, 1.16 mmol) in MeOH (50 mL) was added aqueous ammonium (15 mL), and the reaction mixture was stirred at room temperature. After 1 h, TLC indicated the consumption of the starting material. Removal of solvents yielded a yellowish solid, which was taken up in CH$_2$Cl$_2$ and given a water wash. The organic layer was dried over sodium sulfate, and the solvent was evaporated in vacuo to get a pale yellow solid foam, which was purified by chromatography on silica gel (pre-neutralized with Et$_3$N). Compound 9 eluted in MeOH (3%) in CH$_2$Cl$_2$. Yield: 0.56 g, 87%. $^1$H NMR (500 MHz, CDCl$_3$): $\delta$: 1.84 (s, 3H), 3.25 (s, 1H), 3.48 (s, 3H), 3.74 (s, 3H), 3.76 (s, 3H), 3.68–3.79 (m, 2H), 4.01–4.08 (m, 1H), 4.19–4.23 (m, 1H), 5.51 (d, $J = 1.9$ Hz, 1H), 6.82 (dd, $J = 2.4$, 8.9 Hz, 4H), 7.19–7.34 (m, 8H), 7.41–7.45 (m, 2H), 7.71 (d, $J = 1$ Hz, 1H) ppm; $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$: 12.5, 55.2, 59.0, 70.8, 77.3, 78.5, 81.1, 87.3, 90.9, 109.5, 113.3, 127.1, 127.9, 130.2, 133.5, 135.8, 136.5, 144.8, 150.9, 158.8, 164.2 ppm; HRMS: mass calculated for C$_{14}$H$_{23}$O$_9$Na (M + Na)$^+$ 597.2207, observed (M + Na)$^+$ 597.2207.
were subjected to the van transition; the enthalpy change (melting was performed by monitoring the CD intensity at 295 length cuvette using a resolution of 1 nm, bandwidth of 1 nm, experiments. For thrombin-binding studies, oligomers were cleaved from the solid support by treatment with aq. cation by gel filtration through Pharmacia NAP-5 columns, they were purified by column chromatography on silica gel (pre-neutralized by Et3N). The pure compound 10 was eluted by a 1:1 mixture of CH2Cl2/EtOAc containing 1% Et3N. Yield 0.065 g, 60%. 31P NMR (500 MHz, CDCl3) δ: 150.32, 150.93 ppm; HRMS: mass calculated C16H17O6N1NaP (M+ Na)+ 797.3289, observed (M+ Na)+ 797.3286.

Synthesis of Oligonucleotides. Oligonucleotides were synthesized by β-cyanoethyl phosphoramidite chemistry on a Bioautomation Mermade-4 DNA synthesizer. The 2′-Deoxy-3′-phosphoramidites were obtained from Innovassynth Technologies India Ltd. For the 3′-O-Methyl-3′-Deoxy (DPIPA) (0.1 mL, 0.56 mmol) was added to the ice-cooled CH2Cl2 was added to dilute the reaction, and the organic layer indicated complete consumption of the starting material. stirring the reaction mixture at room temperature for 1 h, TLC monitored complete consumption of the starting material. TLC was washed with NaHCO3 and water and then dried over sodium sulfate. Removal of solvents in vacuo a

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The natural logarithm of the folding constant $K$ was plotted against the reciprocal of the temperature, and the data was restricted to the temperature range for which the fraction folded was between 0.03 and 0.97 to ensure the most linear part of the graph, where $K$ was calculated according to the formula

$$K = \frac{\alpha}{1 - \alpha}$$

The slope of the linear fit of this plot is $-\Delta H/R$, while the Y intercept is $\Delta S/R$, where $R$ is the universal gas constant and is equal to 1.98 cal/mol.

UV-Transmittance Measurements for Clotting Inhibition Assay. UV-transmittance studies were performed on a Varian Cary 300 Bio UV-Visible spectrophotometer when the % transmittance change was monitored with time. A 0.1 NIH unit of thrombin (50 NIH/mL, bovine thrombin, Fibroscreen reagent, Tulip Diagnostics (P) Ltd.) was added to an aqueous solution of TBA, TBA-7T, or TBA-9T oligomer (3.7 × 10⁻⁸ M) and incubated for 15 min at 25 °C. The TBA oligomer-thrombin solution was added to a 1.0 mL fibrinogen solution (3.0 × 10⁻⁶ M, Sigma F 3879) in saline (0.85%), and the transmittance at 450 nm was measured at 3 min intervals for a period of 90 min.

Thrombin Time Measurements for Clotting Inhibition. The inhibitory activity of the TBA oligomers on the thrombin-catalyzed conversion of fibrinogen to fibrin was assessed by a thrombin time assay. The time for clot formation at 37 °C was measured on a StaR Max coagulation analyzer (Stago). Each experiment was repeated at least three times; the standard deviation was ±1 s. Bovine thrombin (Tulip Diagnostics, 0.1 NIH unit) was pre-incubated with the TBA oligomer taken at a 0.25 μM concentration for 2 min before addition to fibrinogen from human plasma (2.65 μM, Aldrich F-3897) and measurement of clotting time (thrombin time). For thrombin time measurements with plasma, thrombin (Diagnostics Stago STA-Thrombin, REF 00611, 0.075 NIH unit) was pre-incubated with the TBA oligomer taken at 0.25 μM for 2 min before addition to plasma (HemosIL, Normal Control Assayed 0020003110) and measurement of clotting time according to the manufacturer’s protocol. Each commercial reagent was re-constituted according to the manufacturer’s protocols.

Quadruplex Stability Studies in the Presence of Nucleases. Quadruplex stability studies of TBA and TBA-7T (7.5 μM) were carried out at 37 °C in 100 mM Tris-HCl buffer (pH 8.5) containing 15 mM MgCl2, 100 mM NaCl, and SVPD (6.6 μM/μL) and with 5 μM strand concentration in reaction buffer (pH 4.5) containing 100 mM KCl, 0.5 M sodium acetate, 2.8 M NaCl, and 45 mM ZnSO4 for S1 nuclease (53.4 U/mL). CD spectra were recorded from 320 to 220 nm and the stability of the quadruplex was monitored by the change in the CD amplitude at 295 nm with time after addition of the respective nuclease. The CD amplitude was plotted against the reaction time. When the S1 nuclease stability was monitored by HPLC, S1 nuclease (89 U/mL) was pre-incubated with the TBA oligomer or TBA-7T at 37 °C in reaction buffer (pH 4.5) containing 0.5 M sodium acetate, 2.8 M NaCl, and 45 mM ZnSO4. Aliquots were removed at successive time points and analyzed by RP-HPLC to reveal the quantity of oligonucleotides left intact after heating at 90 °C for 2 min to inactivate the nuclease.

NMR Experiments of Oligomers. 1H NMR spectra of the oligomers were recorded at 4 °C using a Bruker AV 500 NMR spectrometer. The signals corresponding to the solvent water were suppressed. TBA and TBA-7T (100 μM) were dissolved in 150 μL of 10 mM K-phosphate buffer (pH 7.5) containing 100 mM KCl and lyophilized and then taken up in 9:1 (v/v) H2O/D2O.
have given approval to the manuscript. The final version of the manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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**ABBREVIATIONS**

CD, circular dichroism; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; NMR, nuclear magnetic resonance; TLC, thin layer chromatography.

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