# $N^2$- and $C^8$-Substituted Oligodeoxynucleotides with Enhanced Thrombin Inhibitory Activity in Vitro and in Vivo

Gong-Xin He,* Steven H. Krawczyk, S. Swaminathan, Regan G. Shea, Joseph P. Dougherty, Terry Terhorst, Veronica S. Law, Linda C. Griffin, Steven Coutré, and Norbert Bischofberger

*Gilead Sciences, 353 Lakeside Drive, Foster City, California 94404*

*Received July 1, 1997*

2′-Deoxyguanosine (G) analogues carrying various hydrophobic substituents in the $N^2$ and $C^8$ positions were synthesized and introduced through solid-phase synthesis into 15-mer oligodeoxynucleotide, GGTTGGTGTGGTTGG, which forms a chairlike structure consisting of two G-tetrads and is a potent thrombin inhibitor. The effects of the substitutions at $N^2$ and $C^8$ of the G-tetrad-forming G residues on the thrombin inhibitory activity are relatively small, suggesting that these substitutions cause relatively small perturbations on the chairlike structure formed by the oligodeoxynucleotide. Introduction of a benzyl group into $N^2$ of $G_6$ and $G_{11}$ and naphthylmethyl groups into $N^2$ of $G_6$ increased the thrombin inhibitory activity, whereas other substituents in these positions had almost no effect or decreased the activity. Particularly, the oligodeoxynucleotide carrying a 1-naphthylmethyl group in the $N^2$ position of $G_6$ showed an increase in activity by about 60% both in vitro and in vivo. Substitutions on the $N^2$ position of other G residues had little effect or decreased the activity. Introduction of a relatively small group, such as methyl and propynyl, into the $C^8$ positions of $G_1$, $G_5$, $G_{10}$, and $G_{14}$ increased the activity, presumably due to the stabilization of a chairlike structure, whereas introduction of a large substituent group, phenylethynyl, decreased the activity, probably due to the steric hindrance.

## Introduction

Thrombin is a serine protease with multiple functions in hemostasis. Its roles in coronary heart disease and other thrombotic disorders have promoted efforts toward the identification of specific inhibitors[1] and an understanding of their interactions with this enzyme.[2]

A report from our laboratory disclosed the selection of DNA aptamers by screening from a library of $10^{13}$ 96-mer single-stranded oligodeoxynucleotides (ODNs). The consensus sequence GGTTGGTGTGGTTGG (**1**) was found to be a potent inhibitor of thrombin with an $EC_{50}$ of 20 nM in a purified fibrinogen clotting assay.[3] Subsequent studies in cynomolgus monkeys demonstrated that **1** is a potent and rapid-acting anticoagulant with an in vivo half-life of approximately 2 min.[4] Moreover, in an ex vivo model of arterial thrombosis in a rabbit aorta, **1** was found to inhibit clot-bound thrombin, whereas heparin was ineffective.[5] The rapid onset of action, the short in vivo half-life, and the inhibitory activity of clot-bound thrombin suggest that **1** may be medically useful, e.g., as an anticoagulant in cardiopulmonary bypass surgeries.

The three-dimensional solution structure of **1** has been solved using NMR techniques.[6−8] As shown in Figure 1, the chairlike structure adopted by this oligodeoxynucleotide consists of two G-tetrads connected by two TT loops and a single TGT loop. In the structure, two of the diagonally opposed 2′-deoxyguanosine residues within each of the G-tetrads adopt a syn conformation about their glycosidic bonds ($G_1$, $G_{10}$ and $G_5$, $G_{14}$ in Figures 1 and 2).

Previous structure−activity relationship studies[9] show that replacing any of the eight G-tetrad-forming 2′-deoxyguanosine residues ($G_1$, $G_2$, $G_5$, $G_6$, $G_{10}$, $G_{11}$, $G_{14}$, and $G_{15}$) as well as $T_4$ and $T_{13}$ in **1** by an abasic nucleoside analogue residue can significantly decrease the thrombin inhibitory activity of the compound (>500-fold), while replacement of the other residues has a relatively small effect (10-fold decrease for $T_3$, $G_8$, $T_9$, or $T_{12}$) or no effect ($T_7$) on the activity. The variation in activity reflects the magnitude of structural disruption afforded by removal of each base, confirming the importance of the two G-tetrads in maintaining the active form of the molecule.

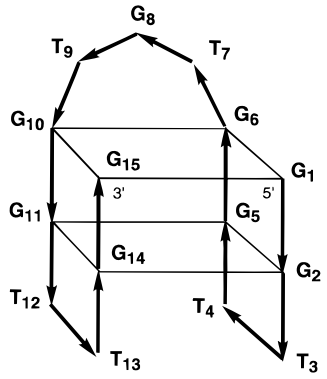



**Figure 1.** Schematic drawing of the NMR solution structure of the 15-mer oligodeoxynucleotide GGTTGGTGTGGTTGG (**1**).

In an effort to increase the potency of **1**, we have pursued attachments which could interact with thrombin and increase the potency. The structure of **1** shows that the G-tetrad-forming 2′-deoxyguanosine (G) residues, which form the core part of the structure, have two positions ($N^2$ and $C^8$) available for attaching one or more groups pointing away from the chairlike structure

S0022-2623(97)00434-2 CCC: $15.00 
Published on Web 05/27/1998

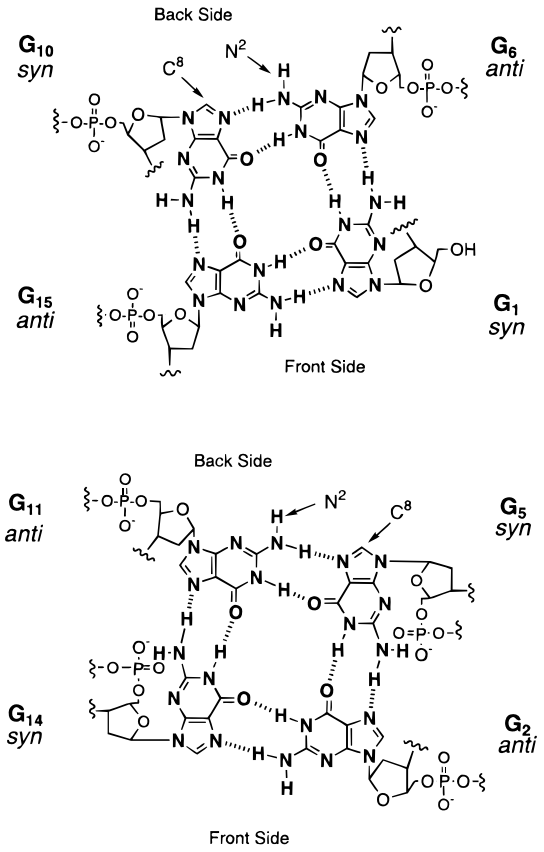



**Figure 2.** Top G-tetrad (top) and bottom G-tetrad (bottom) in the chairlike structure of **1** shown in Figure 1, labeled with the conformation about each glycosidic bond.

(Figure 2). In this paper, we report the results of our studies of ODN analogues, in which modified G residue(s), carrying substituents on the $N^2$ or $C^8$ position, have been introduced into the G-tetrads. It was found that substitutions at certain G residues lead to moderate increases in the thrombin inhibition. The increased activities for the substitutions on $C^8$ positions may be explained by the stabilization of syn conformation of the G residues, while the increased activities for the substitutions on $N^2$ positions may be due to the interaction with thrombin.

## Results

**Synthesis.** $N^2$-Substituted G derivatives were prepared through a 2-fluoro-2′-deoxyguanosine intermediate (Scheme 1).[10] After $O^6$-protection with a *p*-nitrophenylethyl group, the 2-amino group was converted to fluoro through diazotization with *tert*-butyl nitrite in hydrogen fluoride−pyridine.[11] From 2-fluoro intermediate **5**, $N^2$-substituted G derivatives were prepared with the corresponding primary amines.

Synthesis of 8-methyl-2′-deoxyguanosine as well as the corresponding ODNs was reported previously.[9] 8-Propynyl-2′-deoxyguanosine and 8-(phenylethynyl)-2′-deoxyguanosine were prepared through the palladium-mediated coupling of 8-bromo-2′-deoxyguanosine[12] with the corresponding acetylene derivatives (Scheme 2).[13,14]

The modified G monomers were incorporated into ODN analogues with standard solid-phase DNA chemistry utilizing the phosphoramidite method.[15]

**Thrombin Inhibitory Activity.** A prothrombin assay was used to evaluate the thrombin inhibitory activity of the 15-mer analogues. As compared to the purified fibrinogen clotting assay conducted in the previous study,[9] prothrombin time (PT) is measured in human plasma and more directly related to the natural event occurring in vivo.[16] To eliminate the variation caused by the measurements conducted on different days or by using different lots of reagents, relative prothrombin time (relative PT) was determined, which is the activity of the test ODN relative to **1** measured concurrently.

Introduction of a benzyl group into the $N^2$ position of the G residues in **1** led to different effects on the thrombin inhibitory activity (Table 1). Substitutions on $G_6$ (ODN **105**) and $G_{11}$ (ODN **107**) cause a moderate increase in the activity (30−40%), while substitution on $G_{10}$ (ODN **106**) slightly increased the activity (20%). On the other hand, however, substitutions on $G_5$ (ODN **103**) and $G_{14}$ (ODN **108**) significantly decreased the activity, and the substitutions at other positions produced almost no effect. Moreover, introduction of a phenylethyl group or a phenylpropyl group on $G_6$ (ODN **109** or **111**, respectively) or $G_{11}$ (ODN **110** or **112**, respectively) had little effect on the activity.[17]

The 1-naphthylmethyl group on $G_6$ of **113** increased the activity by 60%, while 2-naphthylmethyl group on $G_6$ of **116** gave a 30% increase (Table 2). However, the 4′-biphenylmethyl and 3′-biphenylmethyl on $G_6$ or $G_{11}$ had almost no effect on the activity (ODNs **119**, **120**, **122**, and **123**). For the naphthylmethyl groups and biphenylmethyl groups, double substitutions on both $G_6$ and $G_{11}$ always decreased the activity (ODNs **115**, **118**, **121**, and **124**). Single incorporation of the adamantylmethyl group on $G_6$ or $G_{11}$ (ODN **125** or **126**, respectively) also decreases the activity.

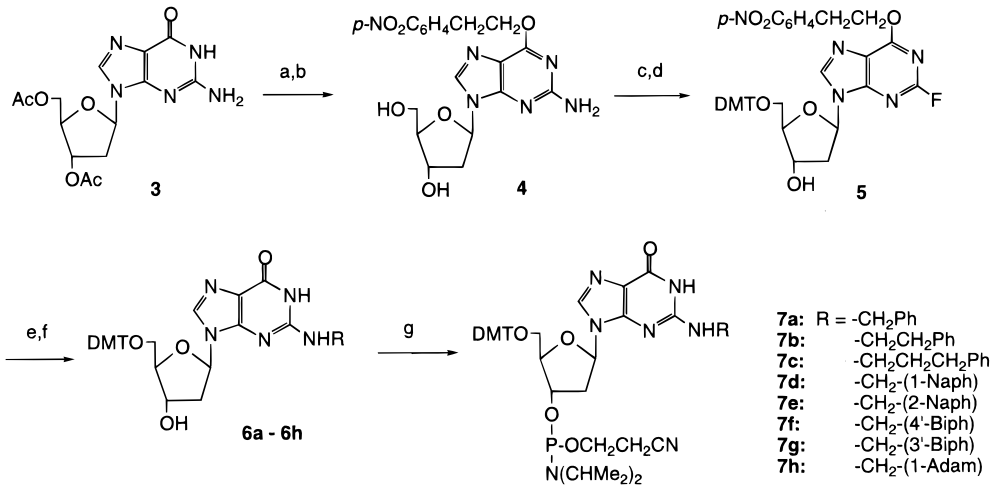
As shown in Table 3, single incorporation of the methyl group at $C^8$ of $G_1$, $G_5$, or $G_{10}$ in **127**, **128**, or **129**, respectively, had almost no effect on the activity. However, **130** carrying one methyl group on $C^8$ of $G_{14}$ showed increased activity. In **131**, when four methyl groups were introduced simultaneously into $G_1$, $G_5$, $G_{10}$, and $G_{14}$, the activity was increased by 60%. Attaching a middle-size propynyl group into $G_1$, $G_5$, $G_{10}$, or $G_{14}$ (ODN **132**, **133**, **134**, or **135**, respectively) resulted in a more pronounced increase of the activity. However, the propynyl substitution lost its advantage over the methyl substitution when all four substitutions were made at once (ODN **136**). Moreover, adding the larger phenylethynyl group showed little effect in single substitution (ODNs **137**, **138**, **139**, or **140**, respectively) and led to decreased activity in the quadruple substitution (ODN **141**).

The increased thrombin inhibitory activity of a substituted ODN was further confirmed in vivo in monkeys. Figure 3 shows the time course of the ODN-mediated anticoagulation (PT) during a constant-rate iv infusion at different concentrations of **1** or **113** which carries 1-naphthylmethyl group on $N^2$ of $G_6$. Both compounds showed a clear dose response, and **113** had an increased activity compared to **1** without losing the rapid-acting and short half-life feature of the anticoagulant.

## Discussion

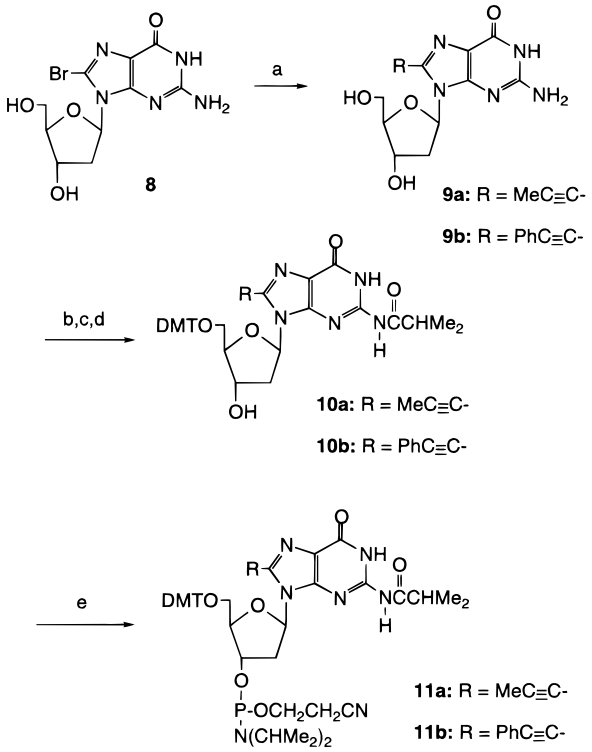
In a previous paper,[9] we have shown that the thrombin inhibitory activity of **1** can be significantly decreased

**Scheme 1**[a]




[a] Reagents: (a) *p*-$NO_2C_6H_4CH_2CH_2OH$, $PPh_3$, DEAD, in 1,4-dioxane; (b) $NH_3$ in MeOH; (c) *t*-Bu-ONO, in 50% Py−HF; (d) DMT-Cl, in Py; (e) R-$NH_2$, in DMF, (f) DBU, in Py; (g) $(Me_2CH)_2NPCl(OCH_2CH_2CN)$, $EtN(CHMe_2)_2$, in $CH_2Cl_2$.

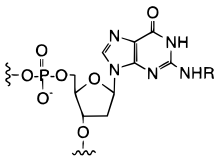
**Scheme 2**[a]




[a] Reagents: (a) MeC≡CH or PhC≡CH, $Et_3N$, Pd catalyst, in DMSO; (b) TMS-Cl, in Py; (c) isobutyric anhydride, in Py, quenched by aq $NH_3$; (d) DMT-Cl, in Py; (e) $EtN(CHMe_2)_2$, $(Me_2CH)_2NPCl(OCH_2CH_2CN)$, in $CH_2Cl_2$.

by replacing one of the eight G-tetrad-forming G residues with a base-modified deoxynucleotide residue, such as 2′-deoxyinosine or 7-deaza-2′-deoxyguanosine. These deoxynucleotide residues are unable to form the hydrogen bond required for the formation of the G-tetrad and consequently cause significant disruption to the chairlike structure. In this study, the effects of the substitutions at $N^2$ and $C^8$ of the G-tetrad-forming G residues of **1** on the thrombin inhibitory activity are relatively small, suggesting that these substitutions cause relatively small perturbations on the chairlike structure formed by the ODN. However, different substitutions

**Table 1.** $N^2$-Substituted G Oligomers and Their Activities: Benzyl, Phenylethyl, and Phenylpropyl Substitutions

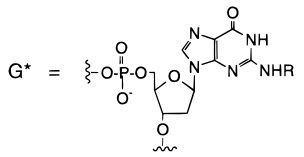
G* = 

| R | No. | Oligomer (5′ → 3′) | Relative PT |
|---|---|---|---|
| -$CH_2$-Ph | 101 | G*GTTGG-TGT-GGTTGG | 1.1 ± 0.1 |
| | 102 | GG*TTGG-TGT-GGTTGG | 0.9 ± 0.1 |
| | 103 | GGTTG*G-TGT-GGTTGG | 0.2 ± 0.1 |
| | 105 | GGTTGG*-TGT-GGTTGG | 1.3 ± 0.1 |
| | 106 | GGTTGG-TGT-G*GTTGG | 1.2 ± 0.1 |
| | 107 | GGTTGG-TGT-GG*TTGG | 1.4 ± 0.1 |
| | 108 | GGTTGG-TGT-GGTTG*G | 0.1 ± 0.1 |
| -$(CH_2)_2$-Ph | 109 | GGTTGG*-TGT-GGTTGG | 1.0 ± 0.1 |
| | 110 | GGTTGG-TGT-GG*TTGG | 0.9 ± 0.1 |
| -$(CH_2)_3$-Ph | 111 | GGTTGG*-TGT-GGTTGG | 1.0 ± 0.1 |
| | 112 | GGTTGG-TGT-GG*TTGG | 1.0 ± 0.1 |

on $N^2$ and $C^8$ cause different effects on the activity, and these effects can be rationalized as follows.

Previous studies using a purified fibrinogen clotting assay showed that introducing a methyl group into the $C^8$ positions of *syn*-G residues of **1** increases the activity, while substitutions at the $C^8$ positions of *anti*-G residues decrease the activity.[9] In the present study, the results obtained from the PT assay conducted in human plasma for ODNs carrying methyl and propynyl substitutions confirm this conclusion. It is known that the presence of a substituent group on the $C^8$ position can stabilize the syn conformation of the G residue.[18] Recently, it was reported that introducing a bromine atom into the $C^8$ position of the *syn*-G residues can stabilize the G-tetrads as well as the chairlike structure.[19] Therefore, the activity increase obtained by introducing a methyl or a propynyl group into the $C^8$ positions of the *syn*-G residues of **1** can be explained by the stabilization of the G-tetrads and consequently the chairlike structure. Since the chairlike structure does not show any change in its UV spectrum upon melting, we have measured the melting point ($T_m$) of the chairlike structure formed by **1** and its derivative **131** using $^1H$ NMR

**Table 2.** N²-Substituted G Oligomers and Their Activities: Naphthylmethyl, Biphenylmethyl, and Adamantylmethyl Substitutions



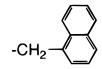
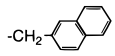
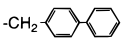
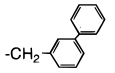
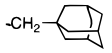
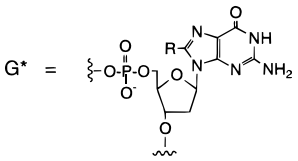
| R | No. | Oligomer (5' → 3') | Relative PT |
|---|---|---|---|
|  | 113 | GGTTGG*-TGT-GGTTGG | 1.6 ± 0.1 |
| | 114 | GGTTGG-TGT-GG*TTGG | 1.1 ± 0.1 |
| | 115 | GGTTGG*-TGT-GG*TTGG | 0.8 ± 0.1 |
|  | 116 | GGTTGG*-TGT-GGTTGG | 1.3 ± 0.1 |
| | 117 | GGTTGG-TGT-GG*TTGG | 1.0 ± 0.1 |
| | 118 | GGTTGG*-TGT-GG*TTGG | 0.9 ± 0.1 |
|  | 119 | GGTTGG*-TGT-GGTTGG | 1.1 ± 0.1 |
| | 120 | GGTTGG-TGT-GG*TTGG | 0.9 ± 0.1 |
| | 121 | GGTTGG*-TGT-GG*TTGG | 0.7 ± 0.1 |
|  | 122 | GGTTGG*-TGT-GGTTGG | 1.2 ± 0.1 |
| | 123 | GGTTGG-TGT-GG*TTGG | 0.7 ± 0.1 |
| | 124 | GGTTGG*-TGT-GG*TTGG | 0.5 ± 0.1 |
|  | 125 | GGTTGG*-TGT-GGTTGG | 0.7 ± 0.1 |
| | 126 | GGTTGG-TGT-GG*TTGG | 0.7 ± 0.1 |

**Table 3.** C⁸-Substituted G Oligomers and Their Activities: Methyl, Propynyl, and Phenylethynyl Substitutions



| R | No. | Oligomer (5' → 3') | Relative PT |
|---|---|---|---|
| Me— | 127 | G*GTTGG-TGT-GGTTGG | 1.2 ± 0.1 |
| | 128 | GGTTG*G-TGT-GGTTGG | 1.1 ± 0.1 |
| | 129 | GGTTGG-TGT-G*GTTGG | 1.1 ± 0.2 |
| | 130 | GGTTGG-TGT-GGTTG*G | 1.4 ± 0.1 |
| | 131 | G*GTTG*G-TGT-G*GTTG*G | 1.6 ± 0.2 |
| MeC≡C— | 132 | G*GTTGG-TGT-GGTTGG | 1.4 ± 0.1 |
| | 133 | GGTTG*G-TGT-GGTTGG | 1.4 ± 0.1 |
| | 134 | GGTTGG-TGT-G*GTTGG | 1.4 ± 0.1 |
| | 135 | GGTTGG-TGT-GGTTG*G | 1.3 ± 0.1 |
| | 136 | G*GTTG*G-TGT-G*GTTG*G | 1.5 ± 0.2 |
| PhC≡C— | 137 | G*GTTGG-TGT-GGTTGG | 0.9 ± 0.1 |
| | 138 | GGTTG*G-TGT-GGTTGG | 1.1 ± 0.1 |
| | 139 | GGTTGG-TGT-G*GTTGG | 0.9 ± 0.1 |
| | 140 | GGTTGG-TGT-GGTTG*G | 1.2 ± 0.1 |
| | 141 | G*GTTG*G-TGT-G*GTTG*G | 0.3 ± 0.1 |

under the same conditions.[6] The melting point was increased from 55 °C for **1** to about 70 °C for **131**, suggesting an improved stability of the chairlike structure formed by **131**.

As shown in Table 3, for the single substitutions, the middle-size propynyl group usually provides better stabilization of the chairlike structure compared to the small methyl group, except for the single methyl substitution at $C^8$ of $G_{14}$ in **130** which had a similar increase in the activity as the single-propynyl substitution. However, further increase in the size of the substituent group as in the large phenylethynyl group does not provide any increase of the stability. The result may be explained by the steric hindrance. Thus, the steric hindrance of the large group diminishes the effect of stabilization by having a substituent group on the $C^8$ position. In the quadruple substitutions, the steric effect of the substituent groups becomes more serious. Therefore, the propynyl substitution showed the same effect as the methyl substitution, while the phenylethynyl substitution showed decreased activity.

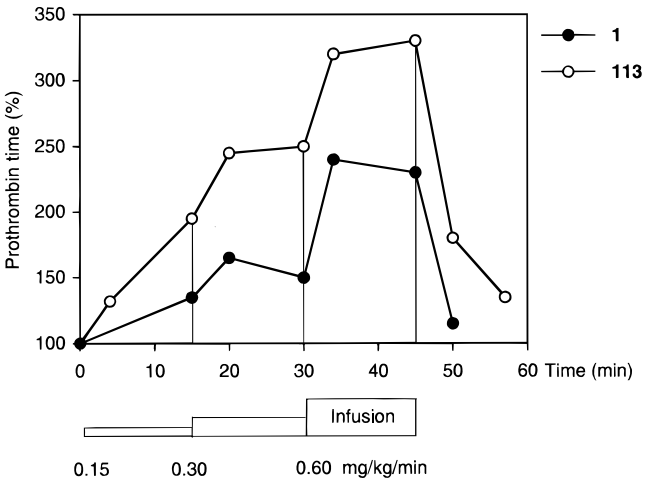



**Figure 3.** Time course of OND-mediated anticoagulation (PT) during a constant-rate iv infusion of 15-mer **1** (●) or modified 15-mer **113** (○) in cynomolgus monkeys. Each OND was infused into two animals over a period of 45 min. The infusion rate was 0.15 mg/kg/min in the time period of 0−15 min, 0.3 mg/kg/min in 15−30 min, and 0.6 mg/kg/min in 30−45 min. Blood samples were collected from each animal at different time points and assayed.

The other attachment site studied is $N^2$ of the G-tetrad-forming G residues.[17] The substitutions on this position provide different effects from the $C^8$ substitutions. As shown in Table 1, the highest increase in the thrombin inhibitory activity was obtained from **105** and **107** (30−40%), which carry a benzyl group on $G_6$ and $G_{11}$, respectively. Since both substituents on $G_6$ and $G_{11}$ are located on the backside of the chairlike structure (Figures 1 and 2), pointing toward the outside, they may have certain interactions with thrombin. In a separate study using neutral formacetal linkage to replace negatively charged phosphodiester linkage, we also found that the backside of the chairlike structure is involved in the binding with thrombin.[20]

On the other hand, however, the substitutions on $G_5$ and $G_{14}$ of **103** and **108**, respectively, decrease the activity. In fact, both substituents on $G_5$ and $G_{14}$ are located in the bottom TT loops where the structure is very crowded due to the phosphate backbone (see bottom G-tetrad in Figure 2). Therefore, substituents on these two positions may destabilize the structure due to the steric hindrance, resulting in the decreased activity.

Since $G_6$ and $G_{11}$ were identified as favorable positions, different attachments on these two positions were studied by either increasing the number of methylene groups or adding another phenyl ring to the attachment. As shown in Tables 1 and 2, however, single substitution of these attachments has a relatively small effect on the activity except for naphthylmethyl groups on $G_6$. For the 1-naphthylmethyl group, the 60% increase of the activity may be explained by the similar interaction with thrombin as achieved by the benzyl group discussed above. However, the double substitution of these groups on both $G_6$ and $G_{11}$ decreases the activity, probably due to the steric hindrance. The same explanation may also

apply to the results obtained from the single substitution of the bulky adamantylmethyl group.

Different from most other thrombin inhibitors, the oligodeoxynucleotides described in this study inhibit the thrombin activity not through binding in the active site. Instead, an anionic binding exosite on the thrombin molecule has been identified as a putative binding site for **1**.[21] Therefore, the inhibition constant ($K_i$) cannot be determined for **1** by measuring the inhibitory effect on the cleavage rate of a small chromogenic substrate. By using surface plasmon resonance technology,[22] we were able to measure the dissociation constant ($K_d$) for **1** and **113**. In this technology, the interaction between the oligodeoxynucleotide and thrombin was monitored by immobilizing thrombin on a sensor chip and injecting **1** across the sensor surface. As a result, $K_d = 1.4$ nM[23] for **1** and $K_d = 0.8$ nM for **113**. The result is consistent with the PT result, although the increase in $K_d$ is also relatively small.

As shown in Figure 3, **131** has increased in vivo thrombin inhibitory activity compared to **1** but maintains a rapid onset of action and a short half-life. In addition, the increase (~60%) in the in vivo activity of **131** is consistent with the increase in the in vitro activity as well as the increase in binding described above. Therefore, like **1**, the in vivo thrombin inhibitory activity of **131** should result from the full-length oligodeoxynucleotide and not from its degradation products. These results are further supported by previous pharmacokinetic studies,[24,25] showing that the rapid clearance of the oligodeoxynucleotide in vivo is mainly due to irreversible tissue uptake and that nuclease degradation in the blood (mainly 3′-exonuclease) only accounts for approximately 22% of the total clearance of **1** in vivo. Therefore, a modification on one of the G residues described in this study will not significantly affect the in vivo pharmacokinetic behavior of the compound.

In conclusion, the effects of the substitutions at $N^2$ and $C^8$ of the G-tetrad-forming G residues on the thrombin inhibitory activity are relatively small, suggesting that these substitutions cause relatively small perturbations on the chairlike structure formed by the ODN. Introducing a small methyl group or a middle-size propynyl group into the $C^8$ position(s) of the *syn*-G residues increases the activity presumably due to the stabilization of the chairlike structure. On the other hand, introducing a 1-naphthylmethyl group into the $N^2$ position of $G_6$ increases the activity by about 60% both in vitro and in vivo probably due to the increased interaction with thrombin.

## Experimental Section

**General.** Column chromatography was performed using silica gel 60 from EM science, and thin-layer chromatography was performed on Kieselgel 60 $F_{254}$ aluminum plates. Solvents used were Burdick & Jackson B&J Chrompure HPLC grade and were dried over 4A molecular sieves. Commercially available chemical reagents were purchased from Aldrich Chemical Co. unless noted otherwise. $^1$H and $^{31}$P NMR spectra were obtained on a General Electric QE-300 at 300.6 and 121.7 MHz, respectively, using tetramethylsilane internal standard for proton and 5% phosphoric acid in $D_2O$ external standard (in capillary) for phosphorus. *J* values are listed in hertz (Hz).

**Relative Prothrombin Time (PT).** The prothrombin time assay (PT) was carried out by the standard method[4,26] on a fibrometer using citrated plasma (Sigma C7916 or freshly spun 1500*g*, 10 min) and thromboplastin/calcium (Sigma T7405) at final concentrations of 2 and 4 $\mu$M for ODNs in plasma. The assay was performed in duplicate at each concentration. The clotting time for the control sample was in the range of 10−13 s. Prothrombin time was the percentage ratio of the clotting time of test ODN to that of the control sample. Relative prothrombin time was the activity of test ODN relative to unmodified **1** measured at the same day and calculated with the following equation:

$$\text{relative PT} = (PT_1 - 100)/(PT_2 - 100)$$

where $PT_1$ represents the prothrombin time of test ODN and $PT_2$ represents the prothrombin time of unmodified **1**. In the experiment, $PT_2$ was about 150 at 2 $\mu$m and 200 at 4 $\mu$m. The results shown in Tables 1−3 are the average values for the data measured at different concentrations of ODNs mentioned above.

**Constant Rate Infusion of ODN and Dose Response in Vivo.** The time course of the ODN-mediated anticoagulation during a constant-rate iv infusion and the dose response of 15-mer **1** or modified 15-mer **113** were studied using cynomolgus monkeys by measuring PT.[4] The animals used, as well as the experiment setting, were the same as those reported previously.[4] Each ODN was infused into two animals over a period of 45 min. The infusion rate was 0.15 mg/kg/min in the time period of 0−15 min, 0.3 mg/kg/min in 15−30 min, and 0.6 mg/kg/min in 30−45 min. Blood samples were collected from each animal at different time points and assayed.

**Synthesis of Oligomers.** ODN analogues were synthesized by using standard solid-phase DNA chemistry on controlled pore glass (CPG) support with the phosphoramidite method[15] on a Biosearch DNA synthesizer. The standard building blocks suitable for the solid-phase synthesis, such as 3′-*O*-[(diisopropylamino)(cyanoethoxy)phosphino]-5′-O-(4,4′-dimethoxytrityl)-$N^2$-isobutyryl-2′-deoxyguanosine and 3′-*O*-[(diisopropylamino)(cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)thymidine, as well as CPG supports loaded with the corresponding building blocks were purchased from Glen Research (Sterling, VA). The CPG supports loaded with the modified building blocks were prepared by standard methods.[27] ODNs were purified by polyacrylamide gel electrophoresis (PAGE) (ca. 1 $\mu$mol) or reversed-phase HPLC (SepTech PRP-1 column) (≥10 $\mu$mol), followed by desalting with a Sephadex NAP-25 column. ODNs were analyzed for purity (≥90%) and chemical integrity by PAGE, ion-exchange HPLC, and base composition analysis. Qualitative PAGE analysis involved inspection of acrylamide gels (25 × 16 × 0.05 cm, 20% acrylamide, 1:19 cross-linked, 7 M urea, Tris/boric acid/EDTA buffer) using UV shadowing or staining with Stains-ALL. Mobilities were compared to that of oligothymidine standards and unmodified 15-mer **1**. Ion-exchange HPLC analysis was performed on a Waters 600 E system with UV detection at 254 nm using a Dionex Nucleopac PA-100 column. The elution gradient was linear from 75 mM LiCl/5 mM LiOH to 1.25 M LiCl/5 mM LiOH in 25 min. To confirm the presence of the expected modified nucleoside components, ODNs were completely digested to nucleoside monomers using snake venom phosphodiesterase, P1 nuclease, and alkaline phosphatase. The digested samples were evaluated via reversed-phase HPLC (Amicon 10 $\mu$m, $C_{18}$, 25 cm × 4.6 mm column, 37 °C, 2% acetonitrile to 35% acetonitrile in 35 min, constant 50 mM monobasic potassium phosphate). After the correction for extinction coefficient differences using the factors of 0.608 and 1.00 for G and T, respectively, the peak areas obtained were used to calculate the relative base composition for each ODN.

ODN extinction coefficients were calculated by the method reported by Tinoco,[28] and unmodified 15-mer **1** has $\epsilon = 143\,300$ based on the method. The $N^2$-substituted and $C^8$-substituted G residues were treated as G in the calculation. Since most of the modified ODNs under consideration contain only one modified base, the error introduced by the treatment can be expected to be less than the systematic error of the assay.

**Synthesis of Monomers. $N^2$-Substituted 2′-Deoxyguanosine Derivatives. 6-*O*-(4-Nitrophenethyl)-2′-deoxyguanosine (4).** A suspension of 2′-deoxyguanosine (3 g, 11.3 mmol) and acetic anhydride (5.0 g, 49 mmol) in 40 mL of pyridine and 30 mL of DMF was heated at 75 °C for 4 h to give a clear solution. TLC showed the completion of the reaction. After cooling, the solution was concentrated to dryness, and the solid residue obtained was washed twice with 2-propanol to give 3.2 g of 3′,5′-di-*O*-acetyl-2′-deoxyguanosine (**3**) (yield, 81%) as a white solid. The structure was confirmed by $^1$H NMR (DMSO-$d_6$).

3′,5′-Di-*O*-acetyl-2′-deoxyguanosine (**3**) (3.2 g, 9.1 mmol) was suspended in 100 mL of dioxane. After triphenylphosphine (3.6 g, 13.7 mmol) and 4-nitrophenethyl alcohol (2.3 g, 13.7 mmoL) were added, the suspension was heated at 80 °C for 30 min. Then, the suspension was cooled to 60 °C, and diethyl azodicarboxylate (2.4 g, 13.7 mmol) was added dropwise. The resultant mixture was heated at 60 °C for another 2 h, and a clear-yellow solution was obtained. After cooling, the solution was concentrated. The residue was dissolved in 300 mL of $CH_2Cl_2$, and the organic solution obtained was washed with 200 mL of water and dried over $Na_2SO_4$. The solution was concentrated, and the residue obtained was purified with silica gel chromatography using $CH_2Cl_2$/MeOH = 95/5 (v/v). A yellow viscous oil was obtained, and $^1$H NMR showed that it contained mainly the desired product, 3′,5′-di-*O*-acetyl-6-*O*-(4-nitrophenethyl)-2′-deoxyguanosine, contaminated by a small amount of triphenylphosphine oxide. The crude product thus obtained was dissolved in 150 mL of 1 M $NH_3$/methanol solution. The solution was stirred at room temperature for 15 h. After the reaction was completed, the solvent was evaporated and the residue was purified with silica gel chromatography using $CH_2Cl_2$/MeOH = 90/10 (v/v) to give 3.0 g of 6-*O*-(4-nitrophenethyl)-2′-deoxyguanosine (**4**) as a pale-yellow foam in 79% yield. $^1$H NMR (DMSO-$d_6$): $\delta$ 8.17 (d, $J$ = 8, 2H, Ar−H), 8.06 (s, 1H, 8-H), 7.61 (d, $J$ = 8, 2H, Ar−H), 6.44 (bs, 2H, NH), 6.19 (t, $J$ = 6.5, 1H, 1′-H), 5.26 (bs, 1H, 3′-OH), 4.98 (bs, 1H, 5′-OH), 4.65 (t, $J$ = 6.5, 2H, $-CH_2-$), 4.34 (m, 1H, 3′-H), 3.81 (m, 1H, 4′-H), 3.4−3.6 (m, 2H, 5′-Hab), 3.24 (t, $J$ = 6.5, 2H, $-CH_2-$), 2.6 and 2.2 (2m, 2H, 2′-Hab).

**5′-*O*-(4,4′-Dimethoxytrityl)-6-*O*-(4-nitrophenethyl)-2-fluoro-2′-deoxyguanosine (5).** Dry pyridine (4 g) was added dropwise at −30 °C to 10 g of hydrogen fluoride−pyridine (containing about 70% HF in pyridine; Aldrich) under stirring to prepare 50% hydrogen fluoride−pyridine. To a suspension of 6-*O*-(4-nitrophenethyl)-2′-deoxyguanosine (**4**) (2 g, 4.8 mmol) in 14 g of 50% hydrogen fluoride−pyridine prepared above at −30 °C was added 98% *tert*-butyl nitrite (0.7 g, 6 mmol) dropwise, and the resultant mixture was stirred at −30 °C for 2 h. After the bubbling stopped, a clear-yellow solution was obtained. The solution was poured into 250 mL of ice−water, and the mixture was extracted with $CH_2Cl_2$ (5 × 100 mL). The solvent used was removed to give 6-*O*-(4-nitrophenethyl)-2-fluoro-2′-deoxyguanosine (2 g, 98%) as a pale-yellow foam, which was used in the subsequent reaction without further purification.

4,4′-Dimethoxytrityl chloride (2.0 g, 5.9 mmol) was added to a solution of 6-*O*-(4-nitrophenethyl)-2-fluoro-2′-deoxyguanosine (2 g, 4.8 mmol) in 30 mL of dry pyridine. The solution was stirred at room temperature for 15 h, and 5 mL of MeOH was added. The resultant solution was concentrated, and the residue obtained was dissolved in 300 mL of $CH_2Cl_2$. After being washed with 100 mL of 5% aqueous $NaHCO_3$ solution, the organic solution was dried over $Na_2SO_4$ and concentrated. The crude product obtained was purified with silica gel chromatography using $CH_2Cl_2$/MeOH/$Et_3N$ = 100/5/1 (v/v) to give 2.5 g of 5′-*O*-(4,4′-dimethoxytrityl)-6-*O*-(4-nitrophenethyl)-2-fluoro-2′-deoxyguanosine (**5**) as a pale-yellow foam with 72% yield. $^1$H NMR ($CDCl_3$): $\delta$ 8.17 (d, $J$ = 8.5, 2H, Ar−H), 8.03 (s, 1H, 8-H), 7.49 (d, $J$ = 8, 2H, Ar−H), 7.1−7.4 (m, 9H, Ar−H), 6.80 (d, $J$ = 8.5, 4H, Ar−H), 6.37 (t, $J$ = 6.5, 1H, 1′-H), 4.83 (t, $J$ = 6.5, 2H, $-CH_2-$), 4.67 (m, 1H, 3′-H), 4.12 (m, 1H, 4′-H), 3.78 (s, 6H, $-OCH_3$), 3.2−3.5 (m, 2H, 5′-Hab), 3.29 (t, $J$ = 6.5, 2H, $-CH_2-$), 2.8 and 2.5 (2m, 2H, 2′-Hab), 1.91 (d, $J$ = 3, 1H, 3′-OH).

**5′-*O*-(4,4′-Dimethoxytrityl)-$N^2$-benzyl-2′-deoxyguanosine (6a).** A solution of 5′-*O*-(4,4′-dimethoxytrityl)-6-*O*-(4-nitrophenethyl)-2-fluoro-2′-deoxyguanosine (**5**) (2 g, 2.8 mmol) and benzylamine (3.2 g, 30 mmol) in 50 mL of dry DMF was stirred at room temperature for 24 h. When the reaction was completed, the solution was concentrated under vacuum and the residue obtained was dissolved in 300 mL of $CH_2Cl_2$. After being washed with 100 mL of 5% aqueous $NaHCO_3$ solution, the organic solution was dried over $Na_2SO_4$ and concentrated. The crude product obtained was purified with silica gel chromatography using $CH_2Cl_2$/MeOH/$Et_3N$ = 95/5/1 (v/v) to give 1.9 g of 5′-*O*-(4,4′-dimethoxytrityl)-6-*O*-(4-nitrophenethyl)-$N^2$-benzyl-2′-deoxyguanosine as a pale-yellow foam in 84% yield.

5′-*O*-(4,4′-Dimethoxytrityl)-6-*O*-(4-nitrophenethyl)-$N^2$-benzyl-2′-deoxyguanosine (1.9 g, 2.3 mmol) was dissolved in 30 mL of dry pyridine. After 2.3 g of DBU was added, the solution obtained was stirred at room temperature for 15 h. Then, the solution was concentrated under vacuum, and the residue obtained was dissolved in 300 mL of $CH_2Cl_2$. After being washed with 100 mL of 0.5 M aqueous citric acid solution and then 100 mL of 5% aqueous $NaHCO_3$ solution, the organic solution was dried over $Na_2SO_4$ and concentrated. The crude product obtained was purified with silica gel chromatography using $CH_2Cl_2$/MeOH/$Et_3N$ = 90/10/1 (v/v) to give 1.1 g of 5′-*O*-(4,4′-dimethoxytrityl)-$N^2$-benzyl-2′-deoxyguanosine (**6a**) as a white-yellow foam in 73% yield. $^1$H NMR (DMSO-$d_6$): $\delta$ 10.59 (s, 1H, NH), 7.78 (s, 1H, 8-H), 7.1−7.4 (m, 9H, Ar−H), 6.80 (m, 5H, NH + Ar−H), 6.17 (t, $J$ = 6, 1H, 1′-H), 5.29 (d, $J$ = 4, 1H, 3′-OH), 4.33 (m, 3H, 3′-H, N−$CH_2$−Ph), 3.91 (m, 1H, 4′-H), 3.70 (s, 6H, $-OCH_3$), 3.0−3.3 (m, 2H, 5′-Hab), 2.7 and 2.2 (2m, 2H, 2′-Hab).

Compounds **6b**−**h** were prepared with the same procedure as above using compound **5** and 10 equiv of the corresponding amines. The amine used, the product yield, and the $^1$H NMR data of the product are as follows.

**5′-*O*-(4,4′-Dimethoxytrityl)-$N^2$-(2-phenylethyl)-2′-deoxyguanosine (6b).** Phenethylamine; yield: 85%. $^1$H NMR (DMSO-$d_6$): $\delta$ 7.79 (s, 1H, 8-H), 7.1−7.4 (m, 9H, Ar−H), 6.75 (t, $J$ = 9, 4H, Ar−H), 6.31 (t, $J$ = 5, 1H, NH), 6.21 (t, $J$ = 6, 1H, 1′-H), 5.32 (d, $J$ = 4, 1H, 3′-OH), 4.40 (m, 1H, 3′-H), 3.93 (m, 1H, 4′-H), 3.69 (s, 6H, $-OCH_3$), 3.23 (m, 2H, N−$CH_2$−), 3.0−3.2 (m, 2H, 5′-Hab), 2.71 (t, $J$ = 8, 2H, $-CH_2-$Ph), 2.8 and 2.2 (2m, 2H, 2′-Hab).

**5′-*O*-(4,4′-Dimethoxytrityl)-$N^2$-(3-phenylpropyl)-2′-deoxyguanosine (6c).** 3-Phenylpropylamine; yield: 62%. $^1$H NMR (DMSO-$d_6$): $\delta$ 10.42 (bs, 1H, NH), 7.78 (s, 1H, 8-H), 7.1−7.4 (m, 9H, Ar−H), 6.78 (t, $J$ = 9, 4H, Ar−H), 6.30 (bs, 1H, NH), 6.17 (t, $J$ = 6, 1H, 1′-H), 5.31 (d, $J$ = 4, 1H, 3′-OH), 4.40 (m, 1H, 3′-H), 3.93 (m, 1H, 4′-H), 3.70 (s, 6H, $-OCH_3$), 3.0−3.2 (m, 4H, N−$CH_2$−, 5′-Hab), 2.56 (t, $J$ = 8, 2H, $-CH_2-$Ph), 2.7 and 2.2 (2m, 2H, 2′-Hab), 1.7 (m, 2H, $-C-CH_2-C-$).

**5′-*O*-(4,4′-Dimethoxytrityl)-$N^2$-(1-naphthylmethyl)-2′-deoxyguanosine (6d).** 1-Naphthylmethylamine; yield: 89%. $^1$H NMR (DMSO-$d_6$): $\delta$ 10.45 (bs, 1H, NH), 7.8−8.1 (m, 3H, Ar−H), 7.82 (s, 1H, 8-H), 7.1−7.6 (m, 13H, Ar−H), 6.83 (bs, 1H, NH), 6.76 (t, $J$ = 9, 4H, Ar−H), 6.22 (t, $J$ = 6, 1H, 1′-H), 5.26 (d, $J$ = 4, 1H, 3′-OH), 4.7−4.9 (m, 2H, −N−$CH_2$−Ar), 4.28 (m, 1H, 3′-H), 3.91 (m, 1H, 4′−H), 3.68 (s, 6H, $-OCH_3$), 3.0−3.2 (m, 2H, 5′-Hab), 2.7 and 2.2 (2m, 2H, 2′-Hab).

**5′-*O*-(4,4′-Dimethoxytrityl)-$N^2$-(2-naphthylmethyl)-2′-deoxyguanosine (6e).** 2-Naphthylmethylamine; yield: 78%. $^1$H NMR (DMSO-$d_6$): $\delta$ 10.68 (bs, 1H, NH), 7.8−8.0 (m, 3H, Ar−H), 7.80 (s, 1H, 8-H), 7.1−7.6 (m, 13H, Ar−H), 6.92 (t, $J$ = 5, 1H, NH), 6.77 (t, $J$ = 9, 4H, Ar−H), 6.19 (t, $J$ = 6, 1H, 1′-H), 5.30 (d, $J$ = 4, 1H, 3′-OH), 4.52(d, $J$ = 5, 2H, −N−$CH_2$−Ar), 4.34 (m, 1H, 3′-H), 3.92 (m, 1H, 4′-H), 3.69 (s, 6H, $-OCH_3$), 3.0−3.2 (m, 2H, 5′-Hab), 2.7 and 2.2 (2m, 2H, 2′-Hab).

**5′-*O*-(4,4′-Dimethoxytrityl)-$N^2$-(4-biphenylmethyl)-2′-deoxyguanosine (6f).** 4-Biphenylmethylamine; yield: 73%. $^1$H NMR (DMSO-$d_6$): $\delta$ 10.64 (bs, 1H, NH), 7.80 (s, 1H, 8-H), 7.1−7.7 (m, 18H, Ar−H), 6.84 (t, $J$ = 5, 1H, NH), 6.78 (t, $J$ =

9, 4H, Ar−H), 6.19 (t, $J$ = 6, 1H, 1′-H), 5.30 (d, $J$ = 4, 1H, 3′-OH), 4.40 (d, $J$ = 5, 2H, −N−$CH_2$−Ar), 4.36 (m, 1H, 3′-H), 3.93 (m, 1H, 4′-H), 3.69 (s, 6H, −$OCH_3$), 3.0−3.2 (m, 2H, 5′-Hab), 2.7 and 2.2 (2m, 2H, 2′-Hab).

**5′-*O*-(4,4′-Dimethoxytrityl)-*N*$^2$-(3-biphenylmethyl)-2′-deoxyguanosine (6g).** 3-Biphenylmethylamine; yield: 42%. $^1$H NMR ($CDCl_3$): $\delta$ 12.2 (bs, 1H, NH), 7.9 (bs, 1H, NH), 7.49 (s, 1H, 8-H), 7.0−7.4 (m, 18H, Ar−H), 6.74 (t, $J$ = 9, 4H, Ar−H), 6.09 (m, 1H, 1′-H), 4.48 (m, 2H, −N−$CH_2$−Ar), 4.33 (m, 1H, 3′-H), 3.95 (m, 1H, 4′-H), 3.70 (s, 6H, −$OCH_3$), 3.6 and 3.2 (2m, 2H, 5′-Hab), 2.5 and 2.1 (2m, 2H, 2′-Hab).

**5′-*O*-(4,4′-Dimethoxytrityl)-*N*$^2$-(1-adamantylmethyl)-2′-deoxyguanosine (6h).** 1-Adamantylmethylamine; yield: 86%. $^1$H NMR (DMSO-$d_6$): $\delta$ 10.32 (bs, 1H, NH), 7.77 (s, 1H, 8-H), 7.1−7.4 (m, 9H, Ar−H), 6.78 (t, $J$ = 9, 4H, Ar−H), 6.30 (bs, 1H, NH), 6.17 (t, $J$ = 6, 1H, 1′−H), 5.30 (d, $J$ = 4, 1H, 3′-OH), 4.37 (m, 3H, 3′−H), 3.91 (m, 1H, 4′−H), 3.70 (s, 6H, −$OCH_3$), 3.0−3.2 (m, 2H, 5′-Hab), 2.8 (m, 2H, N−$CH_2$−), 2.7 and 2.2 (2m, 2H, 2′-Hab), 1.4−1.9 (m, 15H, −CH− and −$CH_2$− in ad).

**3′-*O*-[(Diisopropylamino)(cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-*N*$^2$-benzyl-2′-deoxyguanosine (7a).** 2-Cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.8 g, 3.6 mmol) was added dropwise to a solution of 5′-*O*-(4,4′-dimethoxytrityl)-*N*$^2$-benzyl-2′-deoxyguanosine (**6a**) (1.1 g, 1.7 mmol) and *N*,*N*-diisopropylethylamine (0.7 g, 5 mmol) in 30 mL of dry $CH_2Cl_2$ at 0 °C. The solution obtained was stirred at 0 °C for 2 h and at room temperature for another 4 h. Then, 5 mL of methanol was added to the solution. After the mixture was stirred at room temperature for 10 min, 300 mL of $CH_2Cl_2$ was added. The solution obtained was washed with 100 mL of 5% aqueous $NaHCO_3$ solution and dried over $Na_2SO_4$. The solvent was removed under reduced pressure at room temperature. The crude product obtained was purified with silica gel chromatography using $CH_2Cl_2$/MeOH/$Et_3N$ = 95/3/1 (v/v). The white foam product obtained was redissolved in 20 mL of $CH_2Cl_2$, and the solution was added dropwise to 200 mL of hexane under vigorous stirring. The precipitate formed was collected by filtration and dried under vacuum to give 1.1 g of 3′-*O*-[(diisopropylamino)(cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-*N*$^2$-benzyl-2′-deoxyguanosine as a white powder in 75% yield. $^{31}$P NMR (DMSO-$d_6$): $\delta$ 148.40, 148.70.

**3′-*O*-[(Diisopropylamino)(cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-*N*$^2$-(2-phenylethyl)-2′-deoxyguanosine (7b).** 3′-*O*-[(Diisopropylamino)(cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-*N*$^2$-(2-phenylethyl)-2′-deoxyguanosine (**7b**) was prepared from 5′-*O*-(4,4′-dimethoxytrityl)-*N*$^2$-(2-phenylethyl)-2′-deoxyguanosine (**6b**) by the method described above. Yield: 48%. $^{31}$P NMR ($CDCl_3$): $\delta$ 148.44, 148.80.

**3′-*O*-[(Diisopropylamino)(cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-*N*$^2$-(3-phenylpropyl)-2′-deoxyguanosine (7c).** 3′-*O*-[(Diisopropylamino)(cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-N$^2$-(3-phenylpropyl)-2′-deoxyguanosine (**7c**) was prepared from 5′-*O*-(4,4′-dimethoxytrityl)-*N*$^2$-(3-phenylpropyl)-2′-deoxyguanosine (**6c**) by the method described above. Yield: 70%. $^{31}$P NMR ($CDCl_3$): $\delta$ 148.72, 149.13.

**3′-*O*-[(Diisopropylamino)(cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-*N*$^2$-(1-naphthylmethyl)-2′-deoxyguanosine (7d).** 3′-*O*-[(Diisopropylamino)(cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-*N*$^2$-(1-naphthylmethyl)-2′-deoxyguanosine (**7d**) was prepared from 5′-*O*-(4,4′-dimethoxytrityl)-*N*$^2$-(1-naphthylmethyl)-2′-deoxyguanosine (**6d**) by the method described above. Yield: 49%. $^{31}$P NMR ($CDCl_3$): $\delta$ 148.68, 149.05.

**3′-*O*-[(Diisopropylamino)(cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-*N*$^2$-(2-naphthylmethyl)-2′-deoxyguanosine (7e).** 3′-*O*-[(Diisopropylamino)(cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-*N*$^2$-(2-naphthylmethyl)-2′-deoxyguanosine (**7e**) was prepared from 5′-*O*-(4,4′-dimethoxytrityl)-*N*$^2$-(2-naphthylmethyl)-2′-deoxyguanosine (**6e**) by the method described above. Yield: 60%. $^{31}$P NMR ($CDCl_3$): $\delta$ 148.75, 149.05.

**3′-O-[(Diisopropylamino)(cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-*N*$^2$-(4-biphenylmethyl)-2′-deoxyguanosine (7f).** 3′-*O*-[(Diisopropylamino)(cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-*N*$^2$-(4-biphenylmethyl)-2′-deoxyguanosine (**7f**) was prepared from 5′-*O*-(4,4′-dimethoxytrityl)-*N*$^2$-(4-biphenylmethyl)-2′-deoxyguanosine (**6f**) by the method described above. Yield: 56%. $^{31}$P NMR ($CDCl_3$): $\delta$ 148.97, 149.28.

**3′-*O*-[(Diisopropylamino)(cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-*N*$^2$-(3-biphenylmethyl)-2′-deoxyguanosine (7g).** 3′-*O*-[(Diisopropylamino)(cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-*N*$^2$-(3-biphenylmethyl)-2′-deoxyguanosine (**7g**) was prepared from 5′-*O*-(4,4′-dimethoxytrityl)-*N*$^2$-(3-biphenylmethyl)-2′-deoxyguanosine (**6g**) by the method described above. Yield: 50%. $^{31}$P NMR ($CDCl_3$): $\delta$ 148.97, 149.38.

**3′-*O*-[(Diisopropylamino)(cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-*N*$^2$-(1-adamantylmethyl)-2′-deoxyguanosine (7h).** 3′-*O*-[(Diisopropylamino)(cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-*N*$^2$-(1-adamantylmethyl)-2′-deoxyguanosine (**7h**) was prepared from 5′-*O*-(4,4′-dimethoxytrityl)-*N*$^2$-(1-adamantylmethyl)-2′-deoxyguanosine (**6h**) by the method described above. Yield: 80%. $^{31}$P NMR ($CDCl_3$): $\delta$ 148.78, 149.50.

**C$^8$-Substituted 2′-Deoxyguanosine Derivatives. 8-(1-Propynyl)-2′-deoxyguanosine (9a).** A suspension of 8-bromo-2′-deoxyguanosine (**8**) (3.24 g, 9.36 mmol) in 50 mL of DMF containing triethylamine (5 mL, 35.9 mmol), cuprous iodide (0.38 g, 2.0 mmol), and tetrakis(triphenylphosphine)palladium (1.16 g, 1.0 mmol) was saturated with propyne at 5 °C. The mixture was stirred in a sealed flask at room temperature for 72 h and then concentrated. The residue was triturated with 75 mL of methanol, and the solid formed was collected by filtration, washed with methanol and ether, and dried under vacuum to yield 2.28 g (80%) of a tan solid which was contaminated with triphenylphosphine as shown by $^1$H NMR. However, this material was found to be suitable for use in the subsequent reaction. The analytical sample was obtained by chromatography purification on silica gel using methylene chloride/methanol (4:1, v/v) followed by crystallization from water/methanol (4:1, v/v) to give **9a** as an amorphous solid. $^1$H NMR (DMSO-$d_6$): $\delta$ 10.7 (s, 1H, NH), 7.8 (m, 2.5H, contaminant), 6.5 (bs, 2H, $NH_2$), 6.2 (dd, 1H, 1′−H), 5.2 (d, 1H, 3′-OH), 4.8 (t, 1H, 5′-OH), 4.3 (m, 1H, 3′−H), 3.8 (m, 1H, 4′−H), 3.4−3.7 (m, 2H, 5′-Hab), 2.1 and 3.0 (2m, 2H, 2′-Hab), 2.1(s, 3H, propyne $CH_3$). Anal. ($C_{13}H_{15}N_5O_4 \cdot 0.9H_2O$) C, H, N.

**8-(1-Phenylethynyl)-2′-deoxyguanosine (9b).** A suspension of 8-bromo-2′-deoxyguanosine (**8**) (1.66 g, 4.8 mmol) in 25 mL of DMSO containing triethylamine (2.5 mL, 18 mmol) and phenylacetylene (0.7 mL, 6.38 mmoL) was treated with bis-(triphenylphosphine)palladium dichloride (0.2 g, 0.28 mmol). The mixture was heated at 85 °C for 14 h and then concentrated. The residue was triturated with 100 mL of acetonitrile, and the solid formed was collected by filtration and crystallized from 100 mL of water/acetonitrile (9:1, v/v). After filtration, a second crop precipitated from the filtrate, which was combined with the crystallized precipitate, and the combined materials were recrystallized from 75 mL of water/acetonitrile (9:1, v/v) to afford 0.58 g of 8-(1-phenylethynyl)-2′-deoxyguanosine (**9b**) as an amorphous solid in 33% yield. $^1$H NMR (DMSO-$d_6$): $\delta$ 10.9 (s, 1H, NH), 7.5−7.8 (m, 5H, phenyl), 6.6 (bs, 2H, $NH_2$), 6.3 (dd, 1H, 1′−H), 5.4 (d, 1H, 3′-OH), 4.9 (t, 1H, 5′-OH), 4.5 (m, 1H, 3′−H), 3.8 (m, 1H, 4′−H), 3.5−3.7 (m, 2H, 5′-Hab), 2.2 and 3.3 (2m, 2H, 2′-Hab). Anal. ($C_{18}H_{17}N_5O_4 \cdot 1.75H_2O$) C, H, N.

**5′-*O*-(4,4′-Dimethoxytrityl)-*N*$^2$-isobutyryl-8-(1-propynyl)-2′-deoxyguanosine (10a).** A suspension of 8-(1-propynyl)-2′-deoxyguanosine (**9a**) (2.5 g, 8.2 mmol) in 100 mL of pyridine was treated with 15 mL (118 mmol) of chlorotrimethylsilane. The mixture was stirred for 15 min, and then, 5 mL (30 mmol) of isobutyric anhydride was added. After the mixture was stirred for 3 h, 20 mL of water and 10 mL of concentrated ammonium hydroxide were added. The mixture was stirred for 1 h and then concentrated. The residue was triturated with

100 mL of water, and the solid formed was collected by filtration and washed with ethanol (2 × 20 mL) and ether (2 × 20 mL) to afford 2.18 g (71%) of a tan solid. A solution of this solid (2.0 g, 5.33 mmol) in 50 mL of pyridine was treated with 2.16 g (6.38 mmol) of 4,4′-dimethoxytrityl chloride. After the mixture was stirred for 20 min, 20 mL of methanol was added and the mixture was concentrated. The residue was partitioned between 100 mL of ethyl acetate and 100 mL of water. The organic layer was separated, washed with water and brine, dried over sodium sulfate, and then concentrated. The residue was triturated with ether, and the solid formed was collected by filtration to give 2.73 g of 5′-*O*-(4,4′-dimethoxytrityl)-$N^2$-isobutyryl-8-(1-propynyl)-2′-deoxyguanosine (**10a**) as a yellow amorphous solid with 63% yield. $^1$H NMR (DMSO-$d_6$): $\delta$ 11.30 and 12.04 (2bs, 2H, NH), 6.6−7.4 (m, 13H, phenyl), 6.38 (dd, 1H, 1′−H), 5.23 (d, 1H, 3′-OH), 4.44 (m, 1H, 3′−H), 3.97 (m, 1H, 4′−H), 3.65 and 3.67 (2s, 6H, $OCH_3$), 3.39 (m, 2H, 5′-Hab), 2.23 and 3.06 (2m, 2H, 2′-Hab), 2.69 (m, 1H, isobutyryl CH), 2.03 (s, 3H, propyne $CH_3$), 1.07 (m, 6H, isobutyryl $CH_3$).

**5′-*O*-(4,4′-Dimethoxytrityl)-$N^2$-isobutyryl-8-(1-phenylethynyl)-2′-deoxyguanosine (10b).** 5′-*O*-(4,4′-Dimethoxytrityl)-$N^2$-isobutyryl-8-(1-phenylethynyl)-2′-deoxyguanosine (**10b**) was prepared from 8-(1-phenylethynyl)-2′-deoxyguanosine (**9b**) by the method described above as a yellow amorphous solid with 83% yield. $^1$H NMR (DMSO-$d_6$): $\delta$ 11.48 and 12.14 (2bs, 2H, NH), 6.6−7.6 (m, 18H, phenyl), 6.53 (dd, 1H, 1′−H), 5.31 (d, 1H, 3′-OH), 4.49 (m, 1H, 3′−H), 4.02 (m, 1H, 4′−H), 3.68 and 3.69 (2s, 6H, $OCH_3$), 3.06 and 3.40 (2m, 2H, 5′-Hab), 2.37 and 3.12 (2m, 2H, 2′-Hab), 2.75 (m, 1H, isobutyryl CH), 1.12 (m, 6H, isobutyryl $CH_3$).

**3′-*O*-[(Diisopropylamino)(cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-$N^2$-isobutyryl-8-(1-propynyl)-2′-deoxyguanosine (11a).** A solution of 5′-*O*-(4,4′-dimethoxytrityl)-$N^2$-isobutyryl-8-(1-propynyl)-2′-deoxyguanosine (**10a**) (1 g, 1.48 mmol) in 15 mL of methylene chloride and 0.75 mL (5.38 mmol) of triethylamine was treated with 0.75 g (3.37 mmol) of 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite. The mixture was stirred for 20 min, and 1 mL of methanol was added. The resulting mixture was concentrated. The residue obtained was triturated with 2 mL of ethyl acetate, and the solid formed was removed by filtration. The filtrate was concentrated, and the residue was chromatographed on a 2.5 cm 20 cm silica column, first eluting with 500 mL of ethyl acetate (1% triethylamine, v/v) and then with ethyl acetate/methanol (9:1, 1% triethylamine, v/v). The fractions containing the product were pooled and concentrated. The residue was coevaporated with ethyl acetate (3 × 5 mL) and then dissolved in 5 mL of ethyl acetate. This solution was dripped into 200 mL of hexane under vigorous stirring. The resulting precipitate was collected by filtration, washed with hexane, and dried in vacuo to give 0.63 g of 3′-*O*-[(diisopropylamino)(cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-$N^2$-isobutyryl-8-(1-propynyl)-2′-deoxyguanosine (**11a**) as a white amorphous solid with 21% yield. $^1$H NMR (DMSO-$d_6$): $\delta$ 11.2 and 12.1 (2bs, 2H, NH), 6.6−7.6 (m, 13H, phenyl), 6.4 (m, 1H, 1′−H), 4.5 (m, 1H, 3′−H), 4.1 (m, 1H, 4′−H), 3.7 (s, 6H, $OCH_3$), 3.2−3.6 (2m, 4H, 5′-Hab, $-OCH_2CH_2CN$), 2.6 and 2.7 (2m, 3H, isobutyryl CH, $-OCH_2CH_2CN$), 2.4 and 3.2 (2m, 2H, 2′-Hab), 2.0 (s, 3H, propyne $CH_3$), 0.9−1.2 (m, 18H, isobutyryl $CH_3$, isopropyl $CH_3$).

**3′-*O*-[(Diisopropylamino)(cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-$N^2$-isobutyryl-8-(1-phenylethynyl)-2′-deoxyguanosine (11b).** 3′-*O*-[(Diisopropylamino)(cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-$N^2$-isobutyryl-8-(1-phenylethynyl)-2′-deoxyguanosine (**11b**) was prepared from 5′-*O*-(4,4′-dimethoxytrityl)-$N^2$-isobutyryl-8-(1-phenylethynyl)-2′-deoxyguanosine (**10b**) by the method described above as a white amorphous solid in 25% yield. $^1$H NMR (DMSO-$d_6$): $\delta$ 11.4 and 12.2 (2bs, 2H, NH), 6.6−7.6 (m, 18H, phenyl), 6.5 (m, 1H, 1′−H), 4.5 (m, 1H, 3′−H), 4.2 (m, 1H, 4′−H), 3.7 (s, 6H, $OCH_3$), 3.2−3.6 (2m, 4H, 5′-Hab, $-OCH_2CH_2CN$), 2.6 and 2.7 (2m, 3H, isobutyryl CH, $-OCH_2CH_2CN$), 2.5 and 3.1 (2m, 2H, 2′-Hab), 0.8−1.2 (m, 18H, isobutyryl $CH_3$, isopropyl $CH_3$).

**Acknowledgment.** This work was supported in part by SBIR Grant No. 1R43 HL484311-01. G.-X.H. expresses gratitude to Brian C. Froehler, Gilead Sciences Inc., for his valuable comments and suggestions during the preparation of the manuscript.

## References

(1) Tapparelli, C.; Metternich, R.; Ehrhardt, C.; Cook, N. S. Synthetic Low Molecular Weight Thrombin Inhibitors: Molecular Design and Pharmacological Profile. *Trends Pharmacol. Sci.* **1993**, *14*, 366−376.

(2) Banner, D. W.; Hadavry, P. Crystallographic Analysis at 3.0-Å Resolution of the Binding to Human Thrombin of Four Active Site-Directed Inhibitors. *J. Biol. Chem.* **1991**, *266*, 20085−20093.

(3) Bock, L. C.; Griffin, L. C.; Latham, J. A.; Vermaas, E. H.; Toole, J. J. Selection of Single-Stranded DNA Molecules that Bind and inhibit Human Thrombin. *Nature* **1992**, *355*, 564−566.

(4) Griffin, L. C.; Tidmarsh, G. F.; Bock, L. C.; Toole, J. J.; Leung, L. L. K. In Vivo Anticoagulant Properties of a Novel Nucleotide-Based Thrombin Inhibitor and Demonstration of Regional Anticoagulation in Extracorporeal Circuits. *Blood* **1993**, *81*, 3271−3276.

(5) Li, W.-X.; Kaplan, A. V.; Grant, G. W.; Toole, J. J.; Leung, L. L. K. A Novel Nucleotide-Based Thrombin Inhibitor Inhibits Clot-Bound Thrombin and Reduces Arterial Platelet Thrombus Formation. *Blood* **1994**, *83*, 677−682.

(6) Wang, K. Y.; McCurdy, S.; Shea, R. G.; Swaminathan, S.; Bolton, P. H. A DNA Aptamer Which Binds to and Inhibits Thrombin Exhibits a New Structural Motif for DNA. *Biochemistry* **1993**, *32*, 1899−1904.

(7) Wang, K. Y.; Krawczyk, S. H.; Bischofberger, N.; Swaminathan, S.; Bolton, P. H. The Tertiary Structure of a DNA Aptamer Which Binds to and Inhibits Thrombin Determines Activity. *Biochemistry* **1993**, *32*, 11285−11292.

(8) Macaya, R. F.; Schultze, P.; Smith, F. W.; Roe, J. A.; Feigon, J. Thrombin-Binding DNA Aptamer Forms a Unimolecular Quadruplex Structure in Solution. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 3745−3749.

(9) Krawczyk, S. H.; Bischofberger, N.; Griffin, L. C.; Law, V. S.; Shea, R. G.; Swaminathan, S. Structure−Activity Study of Oligodeoxynucleotides Which Inhibit Thrombin. *Nucleosides Nucleotides* **1995**, *14*, 1109−1116.

(10) Robins, M. J.; Uznanski, B. Nucleic Acid Related Compounds. 34. Non-Aqueous Diazotization with *tert*-Butyl Nitrile. Introduction of Fluorine, Chlorine, and Bromine at C-2 of Purine Nucleosides. *Can. J. Chem.* **1981**, *59*, 2608−2611.

(11) Lee, H.; Hinz, M.; Stezowski, J. J.; Harvey, R. G. Syntheses of Polycyclic Aromatic Hydrocarbon-Nucleoside and Oligonucleotide Adducts Specifically Alkylated on the Amino Functions of Deoxyguanosine and Deoxyadenosine. *Tetrahedron Lett.* **1990**, *31*, 6773−6776.

(12) Bodepudi, V.; Shibutani, S.; Johnson, F. Synthesis of 2′-Deoxy-7,8-Dihydro-8-Oxoguanosine and 2′-Deoxy-7,8-Dihydro-8-Oxoadenosine and their Incorporation into Oligomeric DNA. *Chem. Res. Toxicol.* **1992**, *5*, 608−617.

(13) Koyama, S.; Kumazawa, Z.; Kashimura, N. Synthesis of 6- and 8-Alkynylated Purines and their Ribonucleosides by the Coupling of Halopurines with Alkynes. *Nucleic Acids Res. Symp. Ser.* **1982**, *11*, 41−44.

(14) Hobbs, F. W., Jr. Palladium-Catalyzed Synthesis of Alkynylamino Nucleosides. A Universal Linker for Nucleic Acids. *J. Org. Chem.* **1989**, *54*, 3420−3422.

(15) Sinha, N. D.; Biernat, J.; McManus, J.; Koster, H. Polymer Support Oligonucleotide Synthesis XVIII: Use of $\beta$-Cyanoethyl-N,N- Dialkylamino-/N-Morpholino Phosphoramidite of Deoxynucleosides for the Synthesis of DNA Fragments Simplifying Deprotection and Isolation of the Final Product. *Nucleic Acids Res.* **1984**, *12*, 4539−4557.

(16) White, G. C., II; Marder, V. J.; Colman, R. W.; Hirsh, J.; Salzman, E. W. In *Hemostasis and Thrombosis, Basic Principles and Clinical Practice*, 2nd ed.; Colman, R. W., Hirsh, J., Marder, V. J., Salzman, E. W., Eds.; J. B. Lippincott Co.: Philadelphia, 1982; Chapter 65, pp 1048−1060.

(17) The 15-mer ODN carrying a benzyl group on $N^2$ position of $G_{15}$ was not synthesized in this study. However, the 15-mer ODN carrying a 1-naphthylmethyl group on $N^2$ position of $G_{15}$ was synthesized and tested (data not shown). It has a slightly decreased activity compared to **1**.

(18) Rao, S. N.; Kollman, P. A. Conformations of the 8-Methylated and unmethylated Ribohexamer r(CGCGCG)$_2$. *J. Am. Chem. Soc.* **1986**, *108*, 3048−3053.

(19) Dias, E.; Battiste, J. L.; Williamson, J. R. Chemical Probe for Glycosidic Conformation in Telomeric DNAs. *J. Am. Chem. Soc.* **1994**, *116*, 4479−4480.
(20) He, G.-X.; Williams, J. P.; Postich, M. J.; Swaminathan, S.; Shea, R. G.; Terhorst, T.; Law, V. S.; Mao, C. T.; Coutré, S.; Bischofberger, N. In vitro and in vivo Activities of Oligodeoxynucleotide-Based Thrombin Inhibitors Containing Neutral Formacetal Linkages. *J. Med. Chem.*, submitted.
(21) Paborsky, L. R.; McCurdy, S. N.; Griffin, L. C.; Toole, J. J.; Leung, L. K. The single-stranded DNA Aptamer-binding Site of Human Thrombin. *J. Biol. Chem.* **1993**, *268*, 20808−20811.
(22) Fagerstam, L. G.; Frostell-Karlsson, A.; Karsson, R.; Persson, B.; Ronnberg, I. Biospecific Interaction Analysis Using Surface Plasmon Resonance Detection Applied to Kinetic, Binding Site, and Concentration Analysis. *J. Chromatogr.* **1992**, *597*, 397−410.
(23) Griffin, L. C.; Leung, L. K. Discovery and Characterization of a Thrombin Aptamer Selected from a Combinatorial ssDNA Library. In *Combinatorial Libraries, Synthesis, Screening and Application Potential*; Cortese, R., Ed.; Walter de Gruyter & Co.: Berlin, 1996; Chapter 5, pp 87−112.
(24) Shaw, J.-P.; Fishback, J. A.; Cundy K. C.; Lee, W. A. A novel oligodeoxynucleotide inhibitor of thrombin. I. In vitro metabolic stability in plasma and serum. *Pharm. Res.* **1995**, *12*, 1937−1942.
(25) Lee, W. A.; Fishback, J. A.; Shaw, J.-P.; Bock, L. C.; Griffin, L. C.; Cundy, K. C. A novel oligodeoxynucleotide inhibitor of thrombin. II. Pharmacokinetics in the Cynomolgus Monkey. *Pharm. Res.* **1995**, *12*, 1943−1947.
(26) Simmons, A. *Hematology, A Combined Theoretical and Technical Approach*, 2nd ed.; Butterworth-Heinemann: Newton, MA, 1997; Chapter 20, pp 343−360.
(27) Damha, M. J.; Giannaris, P. A.; Zabarylo, S. V. An Improved Procedure for Derivatization of Controlled-Pore Glass Beads for Solid-Phase Oligonucleotide Synthesis. *Nucleic Acids Res.* **1990**, *18*, 3813−3821.
(28) Borer, P. N. In *CRC Handbook of Biochemistry and Molecular Biology*, 3rd ed.; Fasman, G. D., Ed.; 1975; Vol. 1, p 589.

JM970434D