In Vitro and in Vivo Activities of Oligodeoxynucleotide-Based Thrombin Inhibitors Containing Neutral Formacetal Linkages

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A series of 15-mer oligodeoxynucleotide analogues were synthesized, and their thrombin inhibitory activities in vitro and in vivo were evaluated. These oligodeoxynucleotide analogues share the same sequence (GGTTGGTGTGGTTGG) but have one or more phosphodiester linkages replaced by a neutral formacetal group. The results obtained from monosubstitutions show that no single phosphodiester group is critical for the thrombin inhibitory activity, suggesting that the interaction between the oligodeoxynucleotide and thrombin is based on a multiple-site charge−charge interaction. Analysis of the effects of different phosphodiester replacements indicates that the backside and left side of the chairlike structure formed by the molecule may be involved in binding with thrombin, presumably by having direct contacts with the anion-binding exosite of the enzyme. For the oligodeoxynucleotides containing two noncontiguous formacetal groups, the effect of the disubstitution is the sum of the effects obtained from the corresponding two monosubstitutions. Infusion of an oligodeoxynucleotide containing four formacetal groups into monkeys showed an increased in vivo anticoagulant effect and an extended in vivo half-life compared to the unmodified oligodeoxynucleotide.

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] as well as the understanding of their interactions with this enzyme.[2]

GS-522 is a novel oligodeoxynucleotide, GGTTGGTGTGGTTGG (**1**), discovered through screening of a combinatorial library containing 10^{13} 96-mer single-stranded oligodeoxynucleotides (ODNs).[3] This 15-mer ODN is a potent inhibitor of thrombin in vitro and in vivo with desirable anticoagulant properties.[4,5] NMR studies showed that **1** forms a chairlike structure in solution consisting of two G-tetrads connected by two TT loops and a single TGT loop (Figure 1).[6−8] Both NMR[7] and structure−activity studies[9,10] confirmed the importance of the tertiary structure for the biological activity. The anion-binding exosite on the thrombin molecule has been identified as a putative binding site for **1**.[11]

Controlled intravenous infusion experiments using cynomolgus monkeys demonstrated that **1** is a potent and rapid acting anticoagulant.[4,5] The prolongation of the plasma prothrombin time (PT) is directly proportional to the infusion rate, and the in vivo half-life of **1** is less than 2 min.[4] Detailed pharmacokinetic studies[12,13] conducted in cynomolgus monkeys showed that **1** is rapidly cleared from plasma by tissue uptake. The degradation by nuclease in the blood accounts for only approximately 22% of the total clearance of **1** in vivo.

As a part of the structure−activity relationship study, we were interested in replacing the charged phosphodiester backbone of **1** with a neutral linkage. One goal of the study was to derive analogues with a higher thrombin inhibitory activity, which in practice could result in a lower dose. Toward that goal, we carried out the present study to understand which phosphodiester group is directly involved in the interaction with thrombin. Since **1** binds to thrombin at the anion-

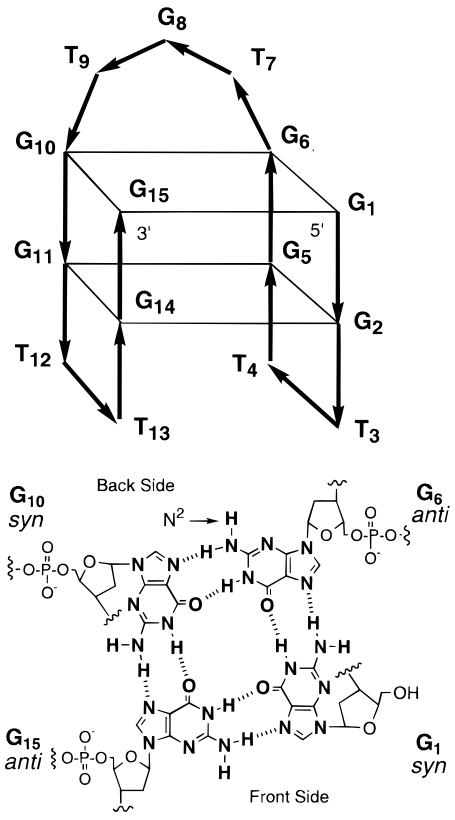

Figure 1. Schematic drawing of the NMR solution structure of GGTTGGTGTGGTTGG (**1**) (top) and the top G-tetrad (bottom) indicating the conformation of the glycosidic bond.

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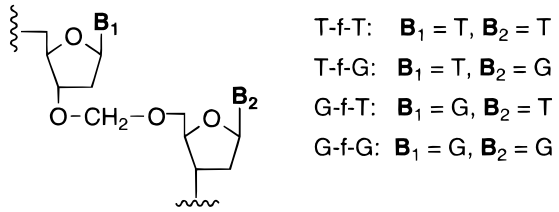
Chart 1[a]



[a] T, thymine; G, guanine; f, formacetal.

binding exosite which contains a number of positively charged amino acid residues,[11] the negatively charged phosphodiester groups in the ODN must play an important role in the binding. Replacing the negatively charged phosphodiester group with a neutral linkage will reveal which phosphodiester group or groups are critical to the interaction. This information will aid in understanding the interaction of **1** with thrombin and may help in designing simpler analogues of **1** with improved properties.

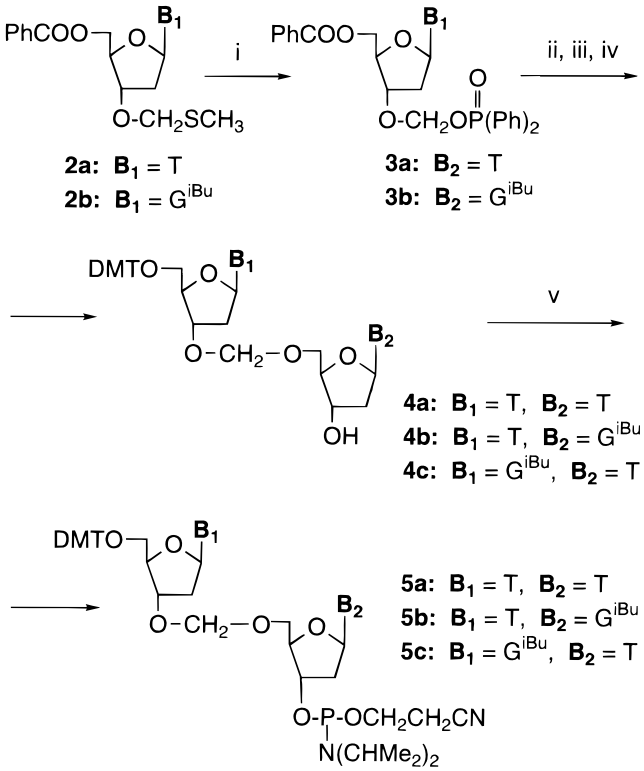
Another goal of this study was to derive analogues with an increased in vivo half-life through structural modifications. The short in vivo half-life makes **1** an ideal anticoagulant for indications such as cardiopulmonary bypass surgeries, where a quick onset of anticoagulation and rapid return to a normal clotting value are desirable. However, analogues of **1** with an increased in vivo half-life could be useful in clinical settings, where an extended anticoagulant effect is necessary (e.g., deep vein thrombosis). Pharmacokinetic studies showed that the short in vivo half-life of **1** is mainly due to rapid tissue uptake.[12,13] Cellular uptake of an ODN occurs mainly through receptor-mediated endocytosis,[14,15] and binding of an ODN to the cell surface is mainly based on charge−charge interactions.[16] Therefore, replacing the negatively charged phosphodiester groups of **1** with a neutral linkage may decrease the tissue uptake and increase the in vivo half-life of **1**.

In the present study, we selected the formacetal group as the neutral linkage to replace the charged phosphodiester group. The formacetal group is achiral, and incorporation of the formacetal linkage into ODNs causes minimal structural perturbations.[17,18] In this paper, we report the synthesis of a series of oligodeoxynucleotide analogues and their thrombin inhibitory activities in vitro and in vivo. These oligodeoxynucleotide analogues have the same sequence as **1** but contain one or more neutral formacetal groups as the replacement for phosphodiester linkages (Chart 1).

Results

Synthesis. The formacetal-linked dimers, including thymidine/thymidine dimer (T-f-T), thymidine/2′-deoxyguanosine dimer (T-f-G), and 2′-deoxyguanosine/thymidine dimer (G-f-T), were prepared through a modified van Boom's procedure shown in Scheme 1. In the original method, van Boom et al.[19] used the condensation of a nucleoside phosphate, 3′-O-CH_2-OP(O)$(OR)_2$, with the 5′-OH group of a second nucleoside in the presence of trimethylsilyl trifluoromethanesulfonate (TMSOTf) to synthesize formacetal-linked pyrimidine−pyrimidine or pyrimidine−purine dinucleotide analogues. We employed a nucleoside phosphinate, 3′-O-CH_2-OP(O)R_2, in place of a nucleoside phosphate in the condensation reaction (Scheme 1). The nucleoside phosphinate is more stable and can be easily isolated and purified.[20]

Scheme 1[a]

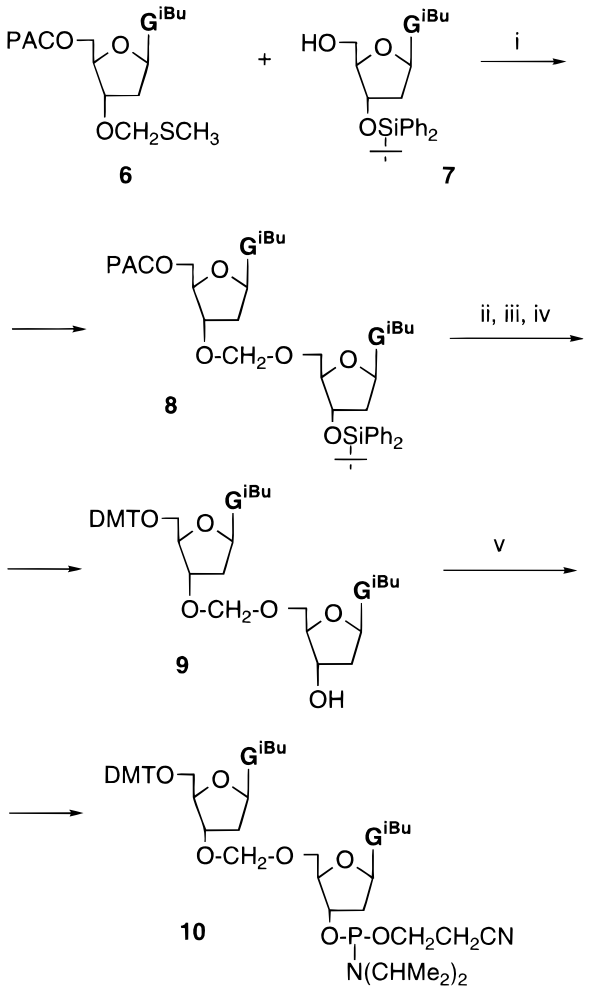


[a] T, thymine; G^{iBu}, N^2-isobutyrylguanine; DMT, 4,4′-dimethoxytrityl. (i) $(Ph)_2P(O)OH$, NIS, CH_2Cl_2/Et_2O; (ii) 3′-*O*-benzoylthymidine or 3′-*O*-benzoyl-N^2-isobutyryl-2′-deoxyguanosine, TMSOTf, CH_2Cl_2, −50 °C; (iii) aq KOH, MeOH−dioxane; (iv) DMT-Cl, Py; (v) $NCC_2H_4OP(Cl)N(CHMe_2)_2$, $EtN(CHMe_2)_2$, CH_2Cl_2.

Neither the phosphate method nor the phosphinate method works in the synthesis of 2′-deoxyguanosine/2′-deoxyguanosine dimer (G-f-G). Under van Boom's conditions using catalytic amounts of TMSOTf,[19] no reaction occurred, while using excess TMSOTf (2−4 equiv) resulted mainly in silylation of the 5′-OH at low temperatures (−30 °C) or in extensive decomposition at higher temperatures (0 °C). By using the method reported by Matteucci et al.,[21,22] which involves the condensation of a nucleoside methylthiomethyl derivative, 3′-O-CH_2SCH_3 (MTM), with the 5′-OH group in the presence of bromine or *N*-bromosuccinimide (NBS), a complicated mixture was obtained. However, we found that in the presence of 2.5 equiv of trifluoromethanesulfonic acid (TfOH), G-f-G can be synthesized in 54% yield through the condensation using *N*-iodosuccinimide (NIS) activation (Scheme 2). The excess acid is used to minimize the effect of the nucleophilic nitrogens of the guanine base moiety. Thus, the condensation reaction of the 3′-O-MTM group with the 5′-OH group is conducted in the presence of 1.5 equiv of NIS and 2.5 equiv of TfOH under anhydrous conditions at low temperature, where the purine nucleotide is stable to the acid. These reaction conditions can also be used to prepare the protected formacetal-linked dimers containing other purine nucleosides and pyrimidine nucleosides.[23]

In this study, dimers T-f-T, T-f-G, and G-f-T were made by the nucleoside phosphinate method. Dimer

Scheme 2[a]



[a] G^{iBu}, N^2-isobutyrylguanine; DMT, 4,4′-dimethoxytrityl; PAC, phenoxyacetyl. (i) NIS, 2.5 equiv of TfOH, CH_2Cl_2/CH_3CN, −30 °C; (ii) NH_3 in MeOH; (iii) DMT-Cl, Py; (iv) $(n\text{-Bu})_4NF$ in THF; (v) $NCC_2H_4OP(Cl)N(CHMe_2)_2$, $EtN(CHMe_2)_2$, CH_2Cl_2.

G-f-G was made by the new methylthiomethyl nucleoside method. The formacetal-linked dimer units were incorporated into ODNs with standard solid-phase DNA chemistry using the phosphoramidite method.[24]

Thrombin Inhibitory Activity. Different from other thrombin inhibitors, the ODNs described in this study are not active site binders but inhibit thrombin by binding to the anionic binding exosite of the thrombin molecule.[11] Binding of the ODN to thrombin does not inhibit thrombin activity to cleave a small chromogenic substrate. Therefore, the inhibition constant (K_i) cannot be determined with the traditional method using a small chromogenic substrate.

In the present study, a prothrombin time (PT) assay was used to evaluate the thrombin inhibitory activity of the ODNs. The assay was conducted by adding thromboplastin and calcium ion to human plasma to generate thrombin from prothrombin, and the thrombin generated catalyzes clotting of fibrinogen.[25,26] In the previous study,[9] a thrombin time (TT) assay was used, where thrombin was added to purified fibrinogen to cause clotting of fibrinogen. Since the PT assay generates thrombin from prothrombin, it is more representative of the in vivo situation. To eliminate the variation caused by the measurements conducted on different days or with different lots of reagents, relative prothrombin time (relative PT) was determined, which is defined as the activity of the test ODN relative to **1** measured concurrently.

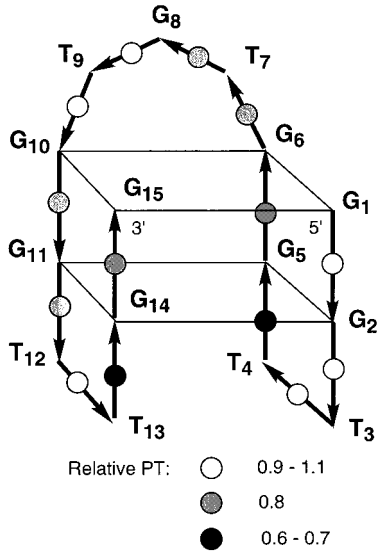


Figure 2. Individual positions and relative PT values of the oligodeoxynucleotides containing one formacetal linkage.

Table 1. Formacetal Monosubstitutions

no.	oligomer	relative PT
101	5′GGT-f-TGGTGTGGTTGG3′	1.1 ± 0.1
102	5′GGTTGGTGTGGT-f-TGG3′	1.1 ± 0.1
103	5′GGTT-f-GGTGTGGTTGG3′	0.7 ± 0.1
104	5′GGTTGGT-f-GTGGTTGG3′	0.8 ± 0.1
105	5′GGTTGGTGT-f-GGTTGG3′	1.1 ± 0.1
106	5′GGTTGGTGTGGTT-f-GG3′	0.6 ± 0.1
107	5′GG-f-TTGGTGTGGTTGG3′	0.9 ± 0.1
108	5′GGTTGG-f-TGTGGTTGG3′	0.8 ± 0.1
109	5′GGTTGGTG-f-TGGTTGG3′	1.1 ± 0.1
110	5′GGTTGGTGTGG-f-TTGG3′	0.8 ± 0.1
111	5′G-f-GTTGGTGTGGTTGG3′	0.9 ± 0.1
112	5′GGTTG-f-GTGTGGTTGG3′	0.8 ± 0.1
113	5′GGTTGGTGTG-f-GTTGG3′	0.8 ± 0.1
114	5′GGTTGGTGTGGTTG-f-G3′	0.8 ± 0.1

Figure 2 and Table 1 show the results obtained from the ODNs where each of the 14 phosphodiester linkages in **1** was successively replaced with a formacetal linkage. Replacing the phosphodiester linkage with a neutral formacetal linkage at T_4-f-G_5 or T_{13}-f-G_{14} decreased the relative PT to 0.7 and 0.6, respectively (**103** and **106**). In other positions, however, replacement of the phosphodiester linkage by a neutral formacetal linkage had only a small effect (relative PT = 0.8) or no effect (relative PT = 0.9−1.1).

The results of multiple substitutions are listed in Table 2. Replacing two phosphodiester linkages simultaneously with two formacetal linkages in the molecule at T_3-f-T_4 and T_{12}-f-T_{13} (**115**) did not affect the relative PT. However, replacing two phosphodiester linkages simultaneously at T_3-f-T_4 and T_{13}-f-G_{14} (**116**) decreased the relative PT to 0.6. The largest decreases were observed for **117**, **119**, and **121**, where the relative PT decreased to 0.3−0.4. These three ODNs contain at least one formacetal linkage in the position of T_4-f-G_5 or T_{13}-f-G_{14}. These are the two positions where a single formacetal substitution caused a relatively large effect on the relative PT as described above. In **122**−**127**, where two phosphodiester linkages between two G units

Table 2. Formacetal Polysubstitutions

substitution	no.	oligomer	relative PT
di	**115**	5′GGT-f-TGGTGTGGT-f-TGG3′	1.1 ± 0.1
	116	5′GGT-f-TGGTGTGGTT-f-GG3′	0.6 ± 0.1
	117	5′GGTT-f-GGT-f-GTGGTTGG3′	0.3 ± 0.1
	118	5′GGTT-f-GGTGT-f-GGTTGG3′	0.7 ± 0.1
	119	5′GGTT-f-GGTGTGGTT-f-GG3′	0.3 ± 0.1
	120	5′GGTTGGT-f-GT-f-GGTTGG3′	0.7 ± 0.1
	121	5′GGTTGGTGT-f-GGTT-f-GG3′	0.4 ± 0.1
	122	5′G-f-GTTG-f-GTGTGGTTGG3′	0.6 ± 0.1
	123	5′G-f-GTTGGTGTG-f-GTTGG3′	0.7 ± 0.1
	124	5′G-f-GTTGGTGTGGTTG-f-G3′	0.7 ± 0.1
	125	5′GGTTG-f-GTGTG-f-GTTGG3′	0.6 ± 0.1
	126	5′GGTTG-f-GTGTGGTTG-f-G3′	0.6 ± 0.1
	127	5′GGTTGGTGTG-f-GTTG-f-G3′	0.8 ± 0.1
tri	**128**	5′GGT-f-TGGT-f-GTGGT-f-TGG3′	0.9 ± 0.1
	129	5′GGT-f-TGGTGT-f-GGT-f-TGG3′	1.1 ± 0.1
tetra	**130**	5′GGT-f-TGGT-f-GT-f-GGT-f-TGG3′	0.5 ± 0.1
	131	5′GGT-f-TGG-f-TG-f-TGGT-f-TGG3′	0.8 ± 0.1

in the molecule were replaced simultaneously by two formacetal linkages, the relative PT decreased to 0.6−0.8. The monosubstitution results showed that replacing one phosphodiester linkage between two G units decreased the relative PT to 0.8−0.9. It seems that the effect of the disubstitution is a simple sum of the effects obtained from the corresponding two individual monosubstitutions.

ODNs **128** and **129** contain three formacetal linkages and have two formacetal linkages simultaneously at T_3-f-T_4 and T_{12}-f-T_{13}, respectively. In addition, **128** contains an additional formacetal linkage at T_7-f-G_8, and **129** contains an additional formacetal linkage at T_9-f-G_{10}. Interestingly, the relative PT values of the two ODNs were not affected by the trisubstitutions. However, tetrasubstitution had some effects on the relative PT value. For example, **130** contains T_7-f-G_8 and T_9-f-G_{10} in addition to T_3-f-T_4 and T_{12}-f-T_{13} and has a relative PT value of 0.5.

In Vivo Anticoagulation Study. In the present study, **130** was chosen for evaluation in an in vivo anticoagulation study in cynomolgus monkeys. The ODN can be synthesized through solid-phase synthesis in the large quantity required by the in vivo study, and since it contains four formacetal groups, **130** was a good candidate for studying the effect of the phosphodiester replacement on the in vivo half-life.

Figure 3 shows the time course of the ODN-mediated anticoagulation (PT) during a constant rate iv infusion of **1** and **130**, in which four phosphodiester linkages were replaced simultaneously by four neutral formacetal linkages at the positions described above. Although **130** had a low relative PT value of 0.5 in vitro, **130** showed an increased in vivo relative PT of 1.7. Moreover, the in vivo activity was also extended as shown by the long tailing in Figure 3. The half-life calculated from the exponential fitting was about 10 min for **130** compared to less than 2 min for **1**. The plasma concentration of **130** in the blood sample collected during the animal study was analyzed by HPLC under the conditions reported previously,[13] and a plasma concentration curve similar to the PT curve was obtained (data not shown), showing an extended half-life of **130** in plasma. The plasma concentration of **130** increased rapidly after the initiation of the infusion and reached a plateau of about 0.1 μM within 2 min under a constant infusion rate of 0.5 mg/kg/min. When the infusion was discontinued,

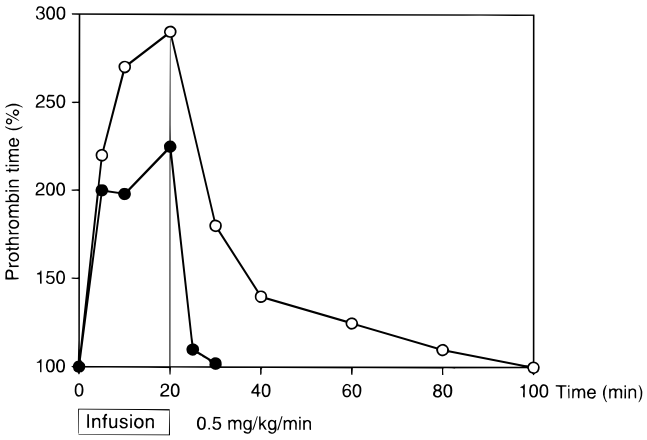


Figure 3. Time course of the ODN-mediated anticoagulation (PT) during a constant rate iv infusion of unmodified **1** (●) or **130** (○) in a cynomolgus monkey. The ODNs were infused into the same animal at an infusion rate of 0.5 mg/kg/min over a period of 20 min. Blood samples were collected from the animal at specific time points and assayed.

the plasma concentration decreased rapidly, and the in vivo half-life of **130** calculated from the plasma concentration curve was 7.6 min, which is significantly increased from 1.5 min for **1** determined previously by the same method.[12,13]

Discussion

Monosubstitution. We have shown that introducing a base-modified deoxynucleotide residue into **1** can significantly affect the thrombin inhibitory activity (relative PT) of the ODN.[9,10] Particularly, modifications at one 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} in Figure 1) produce the largest effect. Modifications stabilizing the tetrad structure increase the thrombin inhibitory activity, whereas modifications destabilizing the structure reduce the activity.[9,10] In the present study, however, replacing any of the 14 phosphodiester linkages in **1** with a neutral formacetal linkage does not dramatically affect the thrombin inhibitory activity. With the monosubstitutions, the maximum decrease was observed for T_{13}-f-G_{14} in **106** (relative PT = 0.6). These results are consistent with the previous observations that the replacement of a phosphodiester linkage with a formacetal linkage in an ODN causes only minimal structural perturbations.[18] These results further suggest that there is not one single phosphodiester group in **1** which is critical for the binding to thrombin and that two or more negatively charged phosphodiester groups may simultaneously be involved. Since the anion-binding exosite of thrombin is a relatively large area consisting of multiple positively charged residues,[11] it can be reasonably concluded that the binding between the ODN and thrombin is based on a multiple-site charge−charge interaction.

As shown in Figure 2, all the phosphodiester groups, where the formacetal substitution causes a decrease in the activity (relative PT ≤ 0.8), are located on the backside and left side of the chairlike structure. Therefore, these two sides may be involved in the binding with thrombin, possibly, by having direct contacts with the anion-binding exosite of the enzyme. We found previously[10] that introducing a 1-naphthylmethyl group into

the N^2 position of G_6 can increase the thrombin inhibitory activity by 60% in both in vitro and in vivo, and the result was explained by the increased interaction with thrombin. The naphthylmethyl group on the N^2 position of G_6 is located on the backside of the chairlike structure pointing toward outside (see Figure 1), further confirming the backside of the chairlike structure as the probable binding site.

The present results (Figure 2) further indicate that the two phosphodiester groups between T_4 and G_5 and between T_{13} and G_{14}, where formacetal substitution causes a relatively large decrease in activity (relative PT = 0.6−0.7), are the most important phosphodiester groups involved in the interaction with thrombin. The phosphodiester groups between T_3 and T_4 and between T_{12} and T_{13} may not directly be involved in binding, since neither monosubstitution nor polysubstitution at these two positions affects activity.

Polysubstitution. A series of ODNs containing two noncontiguous formacetal linkages were evaluated. Due to synthetic difficulties, ODNs containing two or more contiguous formacetal linkages were not prepared. No synergistic effect was observed for the disubstitution on the thrombin inhibitory activity of the ODN. In most cases, the decrease of the relative PT caused by the disubstitution is close to the sum of the decreases caused by the individual monosubstitutions. The lowest relative PT (0.3−0.4) was observed for **117**, **119**, and **121**, in which one or two of the phosphodiester groups between T_4 and G_5 and between T_{13} and G_{14} were replaced, indicating the importance of these two phosphodiester groups.

Two ODNs (**128** and **129**) containing three formacetal linkages and two ODNs (**130** and **131**) containing four formacetal linkages were synthesized. All four ODNs contain both T_3-f-T_4 and T_{12}-f-T_{13}, the two positions where replacement of the phosphodiester group was found to have a minimal effect on activity. Adding another formacetal substitution, T_7-f-G_8 in **128** or T_9-f-G_{10} in **129**, to the two disubstitutions caused little change in the relative PT. However, the tetrasubstitutions in **130** and **131** decrease the activity. These results can be explained by the accumulation of small structural perturbations caused by each substitution.

Since the chairlike structure shown in Figure 1 does not show any change in its UV spectrum upon melting, we have measured, by using ^{1}H NMR, the melting point (T_m) of the chairlike structure formed by **130**. The melting point of **130** was about 50 °C compared to 55 °C for **1** determined previously under the same conditions,[6] suggesting that introducing multiple formacetal groups into the ODN results in a destabilization of the chairlike structure. However, due to the low symmetry of the molecule as well as the complexity of the ^{1}H NMR spectrum, no detailed NMR structural analysis was conducted.

Pharmacokinetics. Detailed in vivo pharmacokinetic studies[13] conducted in cynomolgus monkeys showed that **1** is rapidly cleared from plasma by tissue uptake. The degradation by nucleases in the blood accounts for only approximately 22% of the total clearance of **1** in vivo. Replacing the two phosphodiester linkages at the 3′-end of **1** with nuclease-resistant phosphorothioate linkages has little effect on the in vivo half-life.[13] The conclusion that the short in vivo half-life of the ODN is mainly due to the rapid tissue uptake is further supported by a pilot study in a cardiopulmonary bypass (CPB) model in dogs,[27] showing that the in vivo half-life of **1** was significantly increased by bypassing the lung thereby eliminating a large surface area for the tissue uptake. As shown in Figure 3, **130** demonstrates an increased in vivo PT value and an extended thrombin inhibitory activity compared to **1**, even though its in vitro relative PT is only 0.5. The HPLC analysis of the plasma concentration of **130** showed an extended half-life of **130** in plasma, indicating that the increased in vivo PT value and the extended activity are due to the extended half-life.

Cellular uptake of ODNs has been extensively studied.[28,29] These studies demonstrate that at low ODN concentrations (<0.5 μM), cellular uptake of ODNs occurs mainly through receptor-mediated endocytosis,[14,15] involving receptor binding of ODNs on cell surface as the first step.[30] At high concentrations, both receptor-mediated endocytosis and fluid-phase endocytosis may be involved in the cellular uptake of ODNs.[15,28] The receptor binding of ODNs on cell surface is a rapid and saturatable process and independent of the sequence of ODNs.[31−33] The binding process is mainly based on charge−charge interactions between ODN and receptors.[16] As the number of the charges or the length of the ODN decreases, the binding of ODNs to surface proteins decreases.[34] After endocytosis, most of the ODN is entrapped in endocytic vesicles, and trafficking out of the endocytic vesicles into cytoplasm is an extremely inefficient and slow process.[35,36]

During the in vivo experiment conducted in this study, the plasma concentration of the ODN was about 0.1 μM at a constant infusion rate of 0.5 mg/kg/min, and a receptor-mediated endocytosis process should be the major pathway for tissue uptake of the ODN. Because of the nature of the surface binding and subsequent cellular uptake of ODNs, the slower cellular uptake and the extended in vivo half-life of **130** are probably due to the decreased number of the charges of **130** compared to **1**. This hypothesis is consistent with the observation that the mechanism of cellular uptake was changed from high-efficiency receptor-mediated endocytosis to low-efficiency fluid-phase endocytosis by replacing all negatively charged phosphodiester groups in an ODN with neutral methylphosphonate groups.[37] These data suggest that it is possible to decrease the cellular uptake and increase the in vivo half-life of ODNs by replacing negatively charged phosphodiester groups with neutral linkages.

Conclusions

A series of 15-mer ODNs with one or more negatively charged phosphodiester groups replaced by a neutral formacetal group were synthesized and evaluated for their thrombin inhibitory activity in vitro and in vivo. It was found that more than one of the phosphodiester groups are simultaneously involved in the binding with thrombin. For the ODNs containing two noncontiguous formacetal groups, additive results were obtained for the effect of the substitution on the thrombin inhibitory activity. The in vivo anticoagulation study in monkeys showed that the ODN containing four formacetal groups

shows an increased in vivo PT and extended in vivo half-life compared to the unmodified ODN, suggesting that replacement of negatively charged phosphodiester groups with neutral formacetal groups may decrease the tissue uptake of the ODN.

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 dried over 4A molecular sieves. Commercially available reagents were purchased from Aldrich Chemical unless noted otherwise. ^{1}H NMR and 2D NMR experiments (COSY, HMBC, and HMQC) were conducted on a GE QE-300 300-MHz or Varian Unityplus 500-MHz spectrometer using tetramethylsilane as an internal standard. ^{31}P NMR spectra were obtained on a General Electric QE-300 instrument using 5% phosphoric acid in D_2O as an external standard (in capillary) for phosphorus. *J* values are listed in hertz (Hz).

Relative Prothrombin Time (PT). The prothrombin time (PT) assay was carried out by the standard method[4,25] using citrated plasma (Sigma C7916 or freshly spun at 1500*g*, 10 min) and thromboplastin/calcium (Sigma T7405) at final plasma concentrations of 2 and 4 μM for ODNs. In the assay, the thromboplastin/calcium reagent was added to the plasma in the presence and absence of an inhibitor, and the time required for the plasma to form a clot was recorded on a fibrometer. 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:

$$\text{relative PT} = (\text{PT}_1 - 100)/(\text{PT}_2 - 100)$$

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 μm and 200 at 4 μm. 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 oligodeoxynucleotide-mediated anticoagulation during a constant rate iv infusion of unmodified 15-mer **1** or modified 15-mer **130** was studied using a cynomolgus monkey by measuring PT. The animal used, as well as the experiment setting, was the same as those reported previously.[4] The oligodeoxynucleotides were infused into the same animal at an infusion rate of 0.5 mg/kg/min over a period of 20 min. Blood samples were collected from the animal at specific time points and assayed. The plasma concentration of **130** in the blood samples collected in the experiment was also analyzed by HPLC using the method reported previously.[13]

Synthesis of Oligomers. ODN analogues were synthesized by using the standard solid-phase DNA chemistry on controlled pore glass (CPG) support with the phosphoramidite method[24] on a Biosearch DNA synthesizer. The standard building blocks suitable for the solid-phase synthesis, 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 block (G) were purchased from Glen Research (Sterling, Virginia). The CPG supports loaded with the modified building block were prepared by the standard method.[38] ODNs were purified by polyacrylamide gel electrophoresis (PAGE) (ca. 1 mmol) or reversed-phase HPLC (Sep-Tech PRP-1 column) (≥10 mmol), 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 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 μm, 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 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,[39] and unmodified 15-mer **1** has $\epsilon = 143\,300$ based on the method. The G and T residues present in the formacetal-linked dimers were treated as normal G and T in the calculation. Since most of the modified ODNs under consideration only have one or two phosphodiester linkages replaced by the formacetal linkages, none of the chromophores should be significantly affected and the error introduced by the treatment can be expected to be much less than the systematic error of the assay.

Synthesis of Formacetal-Linked Dimers. Compound 3a. To a solution of **2a** (1 mmol), prepared from thymidine with published method,[40] and diphenylphosphinic acid (0.26 g, 1.2 mmol) in 8 mL of dichloromethane/diethyl ether (2:1, v/v) was added *N*-iodosuccinimide (0.27 g, 1.2 mmol) at room temperature. After 30-min stirring, the mixture was poured into a mixture of 10% $Na_2S_2O_3$ (10 mL) and saturated $NaHCO_3$ (10 mL). The resulting mixture was extracted with CH_2Cl_2 (3 × 10 mL). The organic layer was then washed with brine, dried over $MgSO_4$, and concentrated. Column chromatography using SiO_2/EtOAc provided **3a** as a white foam with 67% yield. ^{1}H NMR ($CDCl_3$): δ 7.4−8.0 (m, 15H, Ar−H), 7.2 (s, 1H, 6-H), 6.24 (dd, J = 5, 6 Hz, 1H, 1′-H), 5.36 (2dd, J = 11, 13 Hz, 2H, $-OCH_2O-$), 4.65 (m, 1H, 3′-H), 4.4 and 4.6 (2dd, J = 3, 11 Hz, 2H, 5′-Hab), 4.29 (m, 1H, 4′-H), 2.2 and 2.5 (2m, 2H, 2′-Hab), 1.65 (s, 3H, CH_3-).

Compound 3b. Compound **3b** was prepared from compound **2b** with the same method as above (yield, 30%). ^{1}H NMR ($CDCl_3$): δ 12.1 (s, 1H, NH), 11.5 (s, 1H, NH), 7.1−7.8 (m, 16H, Ar−H, 8-H), 6.06 (d, J = 8 Hz, 1H, 1′-H), 5.58 (dd, J = 8, 10 Hz, 1H, 3′-H), 5.34 and 5.46 (2dd, J = 5, 9 Hz, 2H, $-OCH_2O-$), 4.46 and 4.63 (2dd, J = 4, 12 Hz, 2H, 5′-Hab), 4.2 (m, 1H, 4′-H), 2.5 and 3.2 (2m, 2H, 2′-Hab), 2.8 (m, 1H, −CH−), 1.13 (t, J = 7 Hz, 6H, $-CH_3$).

Compound 4a. Compound **3a** (0.8 g, 1.4 mmol) and 3′-*O*-benzoylthymidine (0.72 g, 2.1 mmol) were dissolved in 14 mL of dichloromethane and dried over 4A molecule sieves. After 2-h drying, trimethylsilyl trifluoromethanesulfonate (0.31 g, 1.4 mmol) was added dropwise at room temperature. The mixture was stirred for 30 min and filtered through Celite into saturated aqueous $NaHCO_3$. The organic layer was separated, washed with brine, dried over $MgSO_4$, and concentrated to give the crude formacetal-linked dimer. The crude mixture was then redissoved in 20 mL of methanol/dioxane (3:1, v/v), and the solution obtained was cooled to 0 °C. An aqueous KOH solution (1 M, 5 mL) was added to the solution. After 30 min, the reaction solution was neutralized with Dowex acid resin, filtered, and concentrated under reduced pressure. The residue was then coevaporated with ethanol (3 × 5 mL) followed by pyridine (3 × 5 mL). 4,4′-Dimethoxytrityl chloride (0.45 g, 1.3 mmol) was added to the mixture in 12 mL of pyridine, and the solution was stirred at room temperature overnight. The resulting mixture was concentrated under

reduced pressure. The product was purified with column chromatography using $SiO_2/CH_2Cl_2-MeOH-NEt_3$ = 100:5:1 (v/v). Compound **4a** was obtained as a white foam (0.56 g, 57%). ^{1}H NMR ($CDCl_3$): δ 7.66 (s, 1H, 6-H), 7.2−7.4 (m, 10H, 6-H + Ar−H), 6.86 (d, J = 9 Hz, 4H, Ar−H), 6.4 (m, 2H, 1′-H), 4.79 (dd, AB, J = 7 Hz, 2H, $-OCH_2O-$), 4.4 (m, 1H, 3′-H), 4.3 (m, 1H, 3′-H), 4.2 (m, 1H, 4′-H), 4.1 (m, 1H, 4′-H), 3.9 (m, 1H, 5′-Ha), 3.81 (s, 3H, $-OCH_3$), 3.7 (m, 1H, 5′-Hb), 3.55 (d, J = 10 Hz, 1H, 5′-Ha), 3.34 (d, J = 10 Hz, 1H, 5′-Hb), 2.6 (m, 2H, 2′-Ha), 2.4 (m, 2H, 2′-Ha), 2.3 (m, 2H, 2′-Hb), 2.2 (m, 2H, 2′-Hb), 1.90 (s, 3H, $-CH_3$), 1.51 (s, 3H, $-CH_3$).

Compound 4b. Compound **4b** was prepared from compound **3a** and 3′-*O*-benzoyl-N^2-isobutyryl-2′-deoxyguanosine with the same method as above (yield, 48%). ^{1}H NMR ($CDCl_3$): δ 7.73 (s, 1H, 8-H), 7.49 (s, 1H, 6-H), 7.1−7.3 (m, 9H, Ar−H), 6.76 (d, J = 9 Hz, 4H, Ar−H), 6.2 (m, H, 1′-H), 6.1 (m, H, 1′-H), 4.9 (m, 2H, 3′-H), 4.6 (bs, 2H, $-OCH_2O-$), 4.3 (m, 1H, 3′-H), 4.1 (m, 2H, 4′-H), 4.0 (m, 1H, 4′-H), 3.8 (m, 1H, 5′-Ha), 3.71 (s, 6H, $-OCH_3$), 3.6 (m, 1H, 5′-Hb), 3.3 (m, 1H, 5′-Ha), 3.2 (m, 1H, 5′-Hb), 2.8 (m, 1H, −CH−), 2.7 (m, 1H, 2′-Ha), 2.5 (m, 2H, 2′-Hab), 2.2 (m, 1H, 2′-Hb), 1.54 (s, 3H, $-CH_3$), 1.16 (d, J = 7 Hz, 6H, $-CH_3$).

Compound 4c. Compound **4c** was prepared from compound **3b** and 3′-*O*-benzoylthymidine with the same method as above (yield, 10%). ^{1}H NMR ($CDCl_3$): δ 7.80 (s, 1H, 8-H), 7.55 (s, 1H, 6-H), 7.2−7.4 (m, 9H, Ar−H), 6.82 (d, J = 8 Hz, 4H, Ar−H), 6.3 (m, 1H, 1′-H), 6.2 (m, 1H, 1′-H), 4.9 (m, 1H, 3′-H), 4.7 (bs, 2H, $-OCH_2O-$), 4.4 (m, 1H, 3′-H), 4.2 (m, 1H, 4′-H), 4.1 (m, 1H, 4′-H), 3.9 (m, 1H, 5′-Ha), 3.77 (s, 6H, $-OCH_3$), 3.6 (m, 1H, 5′-Hb), 3.4 (m, 1H, 5′-Ha), 3.3 (m, 1H, 5′-Hb), 2.8 (m, 1H, −CH−), 2.7 (m, 1H, 2′-Ha), 2.5 (m, 2H, 2′-Hab), 2.2 (m, 1H, 2′-Hb), 1.62 (s, 3H, $-CH_3$), 1.22 (d, J = 7 Hz, 6H, $-CH_3$).

Compound 5a. 2-Cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (0.66 g, 2.2 mmol) was added dropwise to a solution of compound **4a** (1.8 g, 2.2 mmol) and *N*,*N*-diisopropylethylamine (0.35 g, 2.7 mmol) in 22 mL of dry CH_2Cl_2 at 0 °C. The solution obtained was stirred at 0 °C for 30 min and at room temperature for another 1 h. Methanol, 1 mL, was added to the solution. After the mixture was stirred at room temperature for 10 min, 100 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 column chromatography using SiO_2/EtOAc−MeOH−Et_3N = 100:5:1 (v/v). The white foam product obtained was redissolved in 10 mL of CH_2Cl_2, and the solution was added dropwise to 100 mL of hexane under vigorous stirring. The precipitate formed was collected by filtration and dried under vacuum to give 1.2 g of compound **5a** as a white powder with 58% yield. ^{31}P NMR ($CDCl_3$): δ 148.2, 149.0.

Compound 5b. Compound **5b** was prepared from compound **4b** with the same method as above (yield, 33%). ^{31}P NMR ($CDCl_3$): δ 148.4, 149.1.

Compound 5c. Compound **5c** was prepared from compound **4c** with the same method as above (yield, 18%). ^{31}P NMR ($CDCl_3$): δ 148.9, 149.2.

Compound 8. N^2-Isobutyryl-3′-*O*-methylthiomethyl-5′-*O*-phenoxyacetyl-2′-deoxyguanosine (1.5 g, 2.8 mmol) and N^2-isobutyryl-3′-*O*-*tert*-butyldiphenylsilyl-2′-deoxyguanosine (1.6 g, 2.8 mmol) were dissolved in a solvent mixture containing 20 mL of CH_2Cl_2 and 20 mL of CH_3CN. The solution was dried over 4A molecular sieves (8−12 mesh beads, 3.0 g) for 1 h and cooled to −30 °C. TfOH (1.01 g, 6.7 mmol) was added dropwise followed immediately by NIS (0.72 g, 3.2 mmol). The mixture was stirred at −30 °C for 1 h. The NIS powder was completely dissolved, and the solution turned to dark brown. Triethylamine (1.0 g, 10 mmol) was added slowly at −30 °C to neutralize the acid, and the resulting mixture was then poured into 200 mL of 5% aqueous $Na_2S_2O_3$ solution. The mixture was extracted with CH_2Cl_2 (100 mL × 2), and the organic phase was dried over Na_2SO_4. After the solvent was removed, the solid residue was purified by column chromatography using SiO_2/CH_2Cl_2-EtOAc−MeOH = 50:50:8 (v/v) to give 1.6 g of compound **8** as a pale-yellow foam (yield, 54%). ^{1}H NMR (DMSO-d_6): δ 12.0, 11.7 (2bs, 4H, NH), 8.13, 8.10 (2s, 2H, 8-H), 7.6−6.8 (m, 15H, Ar−H), 6.33, 6.10 (2t, J = 6.5, 7 Hz, 2H, 1′-H), 4.76 (dd, AB, J = 17 Hz, 2H, $-COCH_2OPh$), 4.6 (bs, 2H, $-OCH_2O-$), 4.4 (m, 1H, 3′-H), 4.3 (m, 1H, 3′-H), 4.2 (m, 2H, 5′-Hab), 4.1 (m, 1H, 4′-H), 4.0 (m, 1H, 4′-H), 3.5 (m, 2H, 5′-Hab), 2.8 (m, 3H, 2′-Ha + −CH−), 2.5 (m, 2′-Hb, 1H), 2.3 (m, 2H, 2′-Hab), 1.1 (m, 12H, $-CH_3$), 1.04 (s, 9H, *t*-Bu). High-resolution MS (FAB): calcd for $C_{53}H_{63}N_{10}O_{12}Si$ m/z 1059.4396, found m/z 1059.4378 (M^+ + 1). The formation of 3′-O-CH_2-O-5′ linkage was further confirmed by 2D NMR techniques, including COSY, HMQC, and HMBC (C−H single-bond and multibond correlation).

Compound 9. Compound **8** (1.5 g, 2.8 mmol) was dissolved in 30 mL of CH_2Cl_2. Concentrated NH_3 in MeOH (60 mL) was added, and the solution was stirred at room temperature for 30 min. The disappearance of the starting material was confirmed by TLC using SiO_2/CH_2Cl_2-MeOH = 100:8 (v/v). The reaction solution was then concentrated under reduced pressure and coevaporated with pyridine (3 × 30 mL). The residue obtained was dissolved in 50 mL of dry pyridine. 4,4′-Dimethoxytrityl chloride (1.02 g, 3 mmol) was added, and the resulting solution was stirred at room temperature overnight. After addition of 5 mL of methanol, the solution was stirred for an additional 5 min. Then, the reaction solution was concentrated under reduced pressure. The residue was dissolved in 200 mL of CH_2Cl_2, and the organic solution obtained was washed with 200 mL of 5% aqueous $NaHCO_3$ and dried over Na_2SO_4. Removal of the solvent under reduced pressure gave the crude product, which was further purified by column chromatography using $SiO_2/CH_2Cl_2-MeOH-Et_3N$ = 100:5:1 (v/v) to give 1.5 g of the product as a white foam (yield, 87%). The structure of the product was confirmed by ^{1}H NMR (DMSO-d_6). Next, the product obtained above (1.5 g, 1.2 mmol) was dissolved in 60 mL of THF. A solution of (n-Bu)$_4$NF in THF (1 M, 3 mL, 3 mmol) was added to the above solution. The resulting solution was stirred at room temperature for 30 min. The disappearance of the starting material was confirmed by TLC using $SiO_2/CH_2Cl_2-MeOH-Et_3N$ = 100:10:1 (v/v). Then, the reaction solution was concentrated under reduced pressure. The residue was dissolved in 300 mL of CH_2Cl_2, and the organic solution obtained was washed with 200 mL of 5% aqueous $NaHCO_3$ and dried over Na_2SO_4. After the solvent was removed under reduced pressure, the residue was purified by column chromatography using $SiO_2/CH_2Cl_2-MeOH-Et_3N$ = 100:5:1 (v/v) to give 1.0 g of compound **9** as a white foam (yield, 84%). ^{1}H NMR (DMSO-d_6): δ 11.9, 11.6 (2bs, 4H, NH), 8.13, 8.09 (2s, 2H, 8-H), 7.3−7.1 (m, 9H, Ar−H), 6.8 (d, J = 7 Hz, 2H, Ar−H), 6.7 (d, J = 7 Hz, 2H, Ar−H), 6.20, 6.15 (2t, J = 6.5, 7 Hz, 2H, 1′-H), 5.4 (bs, 1H, OH), 4.76 (dd, AB, J = 7 Hz, 2H, $-OCH_2O-$), 4.4 (m, 1H, 3′-H), 4.3 (m, 1H, 3′-H), 4.0 (m, 1H, 4′-H), 3.9 (m, 1H, 4′-H), 3.69 (s, 6H, $-OCH_3$), 3.6 (m, 1H, 5′-Ha), 3.5 (m, 1H, 5′-Hb), 3.2 (m, 2H, 5′-Hab), 2.8 (m, 3H, 2′-Ha + −CH−), 2.5 (m, 2′-Hb, 1H), 2.3 (m, 2H, 2′-Hab), 1.1 (m, 12H, $-CH_3$).

Compound 10. Compound **10** was synthesized from compound **9** with the same method as for compound **5a**. Compound **10** was obtained as a white powder with 78% yield. ^{31}P NMR (DMSO-d_6): δ 148.3, 147.8.

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