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Design and Synthesis of a Possible Mimic of a Thrombin-Binding DNA Aptamer

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Abstract: A synthesis is presented of the cyclic trimeric d-oligonucleotide 3'-isopropylphosphate **I**, comprising one formacetal and two (3'→ 5')-internucleosidic phosphodiester bonds. The ester linkages connect d-guanosine with the 3' and 5' ends of thymidine and 5-hydroxymethyl-2'-deoxyuridine-3'-isopropylphosphate (HMDUp*i*Pr), respectively. The 5'-end of the thymidine unit is anchored *via* the formacetal bond to the allylic hydroxyl group of HMDUp*i*Pr. The cyclic arrangement of the three d-nucleosides in **I** mimics, as based on molecular modeling, the key structural features of the conformationally constrained $T^7pG^8pT^9p$-domain of the thrombin-binding DNA aptamer d($G^1G^2T^3T^4G^5G^6\underline{T^7G^8T^9}G^{10}G^{11}T^{12}T^{13}G^{14}G^{15}$). Biological evaluation showed that compound **I** did not exhibit anti-thrombin activity. © 1997 Elsevier Science Ltd.

Introduction

The prominent role of serine protease thrombin in thrombosis and haemostasis stimulated extensive research towards the design and synthesis of effective thrombin inhibitors. Recently, Bock *et al.*[1] screened a pool of ~10^{13} synthetic 96-mer oligodeoxynucleotides for their interaction with thrombin using a novel *in vitro* selection/amplification technique. Comparison of the sequences having affinity for thrombin led to the identification of a consensus DNA-15 mer (*i.e.* d($G^1G^2T^3T^4G^5G^6T^7G^8T^9G^{10}G^{11}T^{12}T^{13}G^{14}G^{15}$)). This so-called aptamer inhibits thrombin activity at nanomolar concentrations. Since then a lot of research has been focused on the mode of binding of thrombin with the aptamer. Bock *et al.*[1] and Wu *et al.*[2] showed that the active site of thrombin is not involved in aptamer binding as the aptamer does not inhibit the cleavage of small chromogenic amide substrates. Recent biochemical[2-4] and physical experiments[5-8] revealed that the synthetic 15-mer interacts with the alleged anion-exosite of thrombin. Three-dimensional structure analysis of the aptamer by NMR spectroscopy[5-7] revealed that it adopts a unique folded structure, in which two stacked G-tetrads are connected through a TGT- and two TT-loops (see Fig. 1). A similar folding was reported in a preliminary study[8] on the crystal structure of the aptamer-thrombin complex. The crystallographic analysis also showed that the trimeric $T^7pG^8pT^9p$ domain solely interacts with the anion-exosite of thrombin and adopts a loop conformation in which the 5'-O-T^7 and the 7-C-T^9 are in close proximity (3.4 Å) (see Fig. 2). The latter information goaded us to devise a mimic in which the two

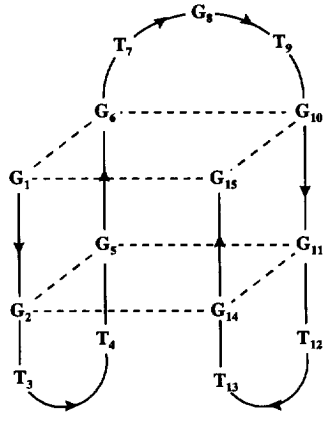

Figure 1: Schematic drawing of the folded 15-mer aptamer.

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thymidines in the trimeric $T^7pG^8pT^9p$-unit are connected by a suitable linker. Molecular modeling studies indicated that this objective could be achieved by replacing of T^9 by 5-hydroxymethyl-2'-deoxyuridine (HMDU) and anchoring the respective primary and allylic hydroxyl in T^7 and HMDU *via* a methylene acetal bond (see Fig. 3). In this way, a loop-structure is created covering a distance between the primary hydroxyl of T^7 and the allylic carbon of HMDU of approximately 2.8 Å. In addition, the (3'-5')-internucleosidic phosphodiester bond between T^9 and G^{10}, the presence of which is essential for thrombin interaction, was replaced by a 3'-isopropyl phosphate. We here report the synthesis of the novel cyclic oligonucleotide **I** (see Fig 3.) containing the key structural features of the aptameric TpGpTp-domain.

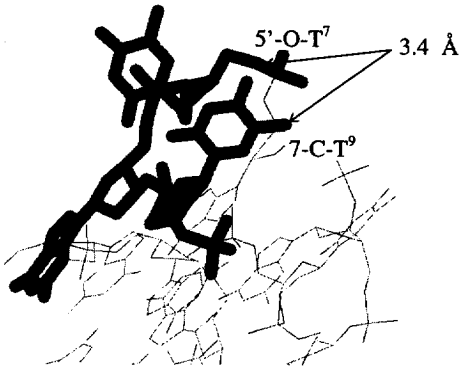


Figure 2: Detail of the crystal structure[8] of the aptamer in the aptamer-thrombin complex. The TpGpTp-domain, which binds with thrombin, is presented as a stick model

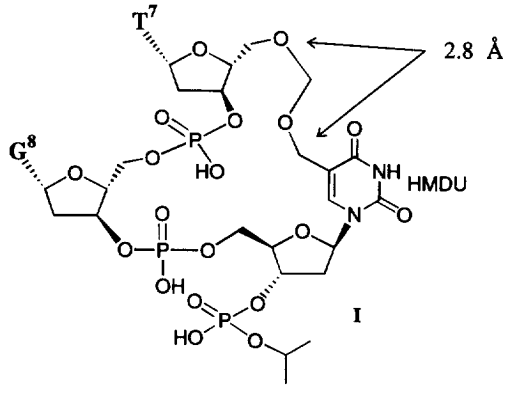
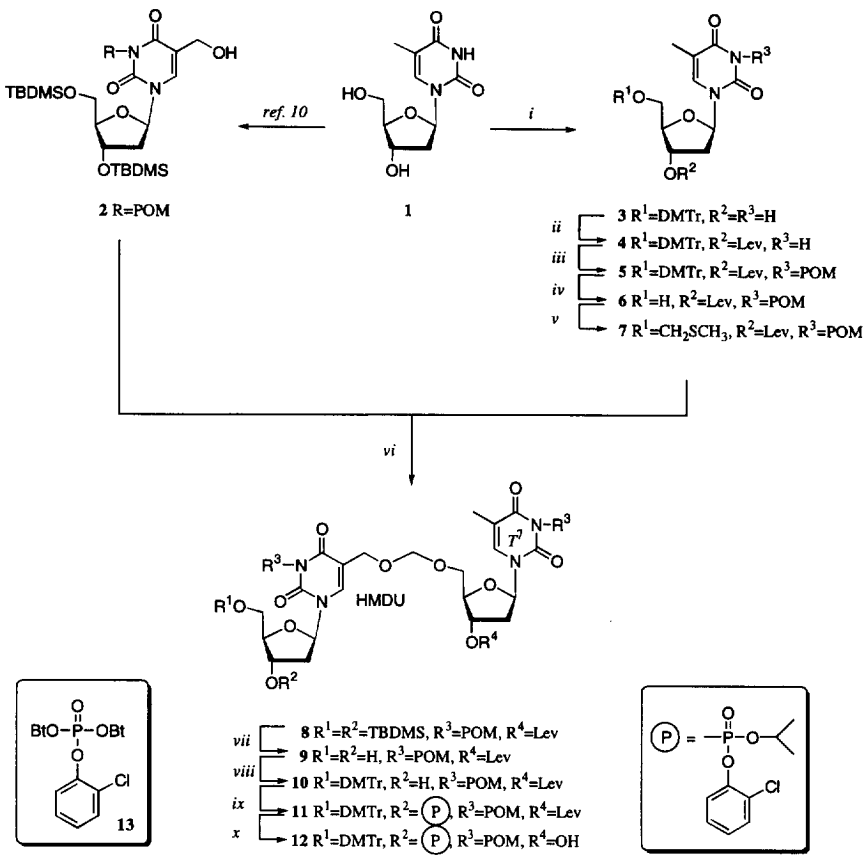


Figure 3. Proposed mimic of the aptameric TpGpTp-domain.

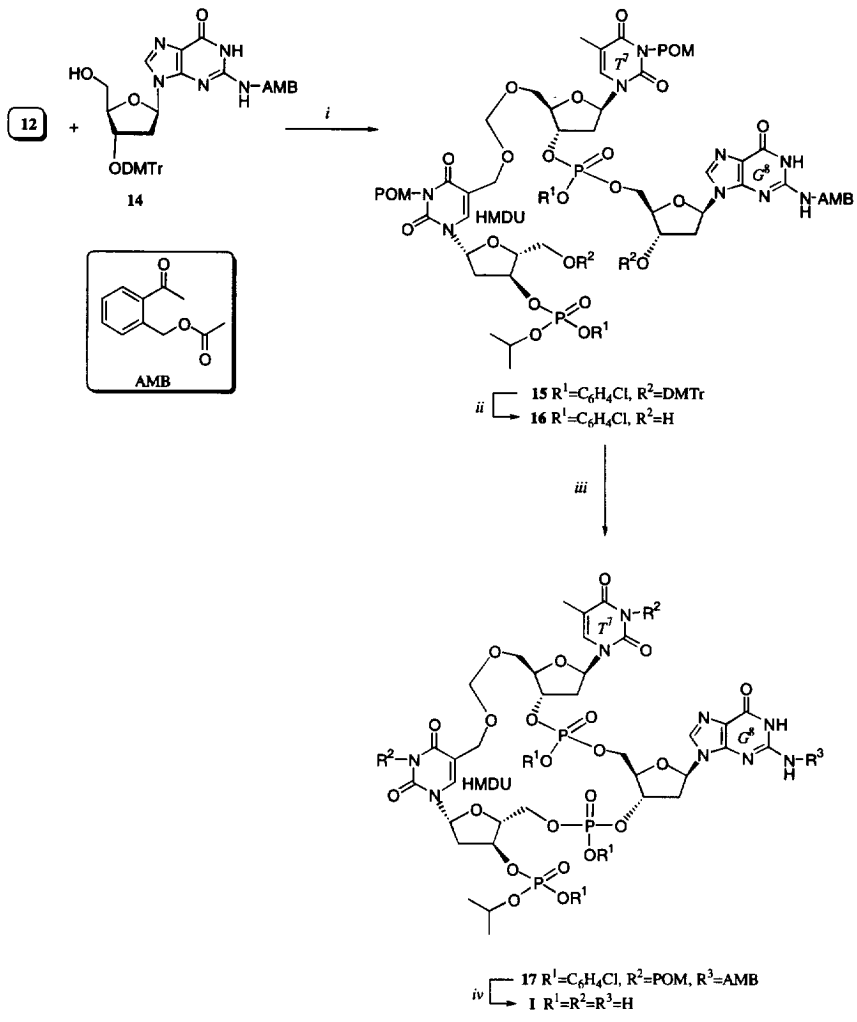
Results and discussion

One of the crucial steps in the assembly of cyclic oligonucleotide **I** entails the introduction of a methylene acetal linkage between the 5'-hydroxyl of T^7 and the allylic hydroxyl function of HMDU. It was established[9] that the formation of the purposive methylene acetal bond could be effected most conveniently starting from 5'-*O*-methylthiomethyl-3'-*O*-levulinoyl-N^3-pivaloyloxymethyl-thymidine (**7**) and known[10] 3',5'-di-*O*-*tert*-butyldimethylsilyl-N^3-pivaloyloxymethyl-5-hydroxymethyl-2'-deoxyuridine (**2**). The requisite donor **7** was readily accessible from **1** by the following five-step procedure. Tritylation of the primary hydroxyl in **1** with 4,4'-dimethoxytrityl chloride (DMTr-Cl) and subsequent acylation of resulting **3** with levulinic acid anhydride gave **4**. Alkylation[11] of **4** in DMF with pivaloyloxymethyl chloride (POM-Cl) in the presence of K_2CO_3, followed by acid treatment of the N-3 protected **5**, led to the isolation of **6**. Transformation of **6** into the thiomethyl ether derivative **7** proceeded as expected by subjecting **6** to dimethylsulfide and benzoylperoxide (BPO) in the presence of 2,6-lutidine[12]. Coupling of **7** with **2** under the agency of *N*-iodosuccinimide (NIS) and catalytic triflic acid (TfOH)[12,13] gave the desired methylene acetal linked dimer **8** in 84% yield. Dimer **8** was converted into **10** by desilylation with triethylamine trihydrofluoride in pyridine[14] and subsequent regioselective tritylation of the



Scheme 1: i) DMTr-Cl, pyridine, 1 h; ii) Lev_2O, pyridine, DMAP, 2 h, 96% (2 steps); iii) POM-Cl, K_2CO_3, DMF, 6 h; iv) 4% *p*-TosOH, MeOH/CH_2Cl_2 (1/1, v/v), 5 min, 72% (2 steps); v) Me_2S (10 equiv), BPO (4 equiv), 2,6-lutidine, CH_3CN/CH_2Cl_2 (1/1, v/v), 2 h, 65%; vi) NIS, *cat.*TfOH, THF, DCE, 5 min, 84%; vii) TEA•3HF, pyridine, 2 h, 89%; viii) DMTr-Cl, pyridine, 1 h, 84%; ix) **13**, 2-propanol, pyridine/dioxane, 3 h, 76%; x) NH_2NH_2, pyridine, AcOH, 5 min;

primary function with DMTr-Cl. Phosphorylation of **10** proceeded smoothly using the well-established bifunctional reagent O-2-chlorophenyl-O,O-*bis*-(benzotriazol-1-yl)phosphate[15] (**13**) to give, after work-up and purification, the homogeneous 3'-(*o*-chlorophenyl)(isopropyl) phosphate derivative **11** (δ p -8.14, -8.37 ppm). Removal of the levulinoyl group in **11** by short treatment with hydrazine in pyridine/acetic acid[16] afforded **12**. The introduction of the (3'→ 5')-internucleosidic linkage between **12** and partially protected d-guanosine derivative **14**, the N^2-2-(acetoxymethyl)benzoyl (AMB) group[17] of which can be removed under mild basic conditions[18], could be readily effected with bifunctional reagent **13** (see Scheme 2). Thus, phosphorylation of **12** with **13**, and coupling of **14** with the *in situ* formed benzotriazol-1-yl phosphate triester of **12**, led to the trimeric derivative **15** (δ p -8.14, -8.19, -8.28, -8.37 ppm). Acidolysis of both DMTr-groups in **15** with *p*-toluenesulfonic acid in dichloromethane/methanol furnished partially deprotected **16** in 85% yield. The linear oligonucleotide **16**



Scheme 2: i) **13**, pyridine, dioxane, 3 h, 63% (2 steps); ii) 4% *p*-TosOH, MeOH/CH_2Cl_2 (1/1, v/v), 5 min, 85%,; iii) **13**, pyridine (6 mM), 54%; iv) a. 0.25 M TBAF, pyridine, H_2O (1/1, v/v); b. 25% NH_4OH, room temperature, 16 h, 63%;

was now converted into the corresponding cyclic and fully protected oligonucleotide **17** according to a well-established protocol[19] devised for the preparation of cyclic oligonucleotides. Thus, **13** was added dropwise to a highly diluted solution (6 mM) of **16** in pyridine. Monitoring of the cyclization by TLC revealed the reaction to be complete (R_f 0 → 0.60, 5% MeOH/CH_2Cl_2) after one hour at 20 °C. Subsequent work-up and purification of the reaction mixture afforded **17** in a 54% yield. The cyclic oligonucleotide **17** was deblocked in a two-step process. Removal of the *o*-chlorophenyl protective groups with tetra-*n*-butylammonium fluoride (TBAF) and subsequent ammonolysis of the POM and AMB protective groups gave completely deblocked cyclic oligonucleotide **I**, which was purified by HW-40 gel filtration and isolated as the sodium salt. The homogeneity of **I** (Na^+-salt) was firmly established by HPLC analysis, ^1H and ^{31}P NMR-spectroscopy as well as ES-MS spectrometry[20]. The existence of the (3'-5')- internucleosidic phosphodiester bond, originating from the

cyclization of **16**, is supported by the two dimensional ^{1}H-^{31}P correlated NMR-spectrum of compound **I** (see Fig. 4). Moreover, the NOE-effects, observed after irradiation of the C5' primary protons of T^{7} in **I**, are in agreement with the presence of the methylene acetal between the primary hydroxyl of T^{7} and the allylic hydroxyl of HMDU[21]. In order to evaluate the extent of thrombin inhibition, compound **I** was tested in a fibrinogen dependent thrombin assay[22]. The outcome of these tests clearly indicated that compound **I** was not active. The biological inactivity of **I** may be explained by the recent findings[23,24] that the two TT-loops (see Fig. 1), instead of the earlier proposed TpGpTp-domain, interact with the anion-exosite of thrombin.

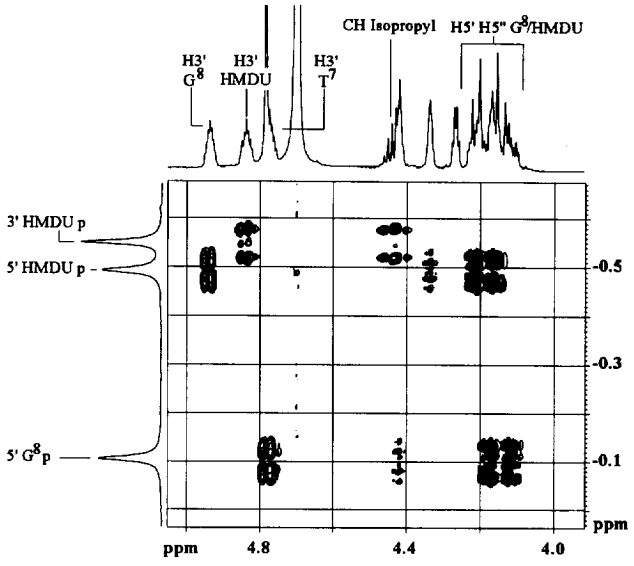


Figure 4: Part of the ^{1}H-^{31}P-NMR spectrum of compound **I**

The design and synthesis of mimics based on this recently attained insight into the mode of interaction of the aptamer with thrombin will be reported in due course.

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