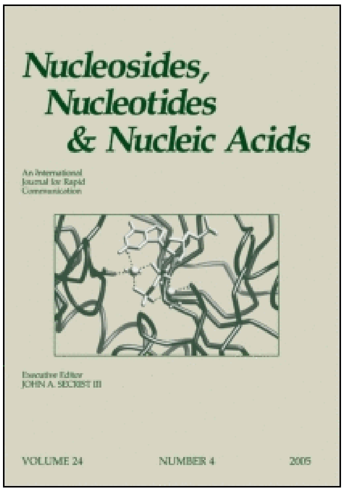

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A MINI-LIBRARY OF TBA ANALOGUES CONTAINING 3′-3′ AND 5′-5′ INVERSION OF POLARITY SITES

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□ *Several researches have been devoted to structure-activity relationship and to post-SELEX modifications of the thrombin binding aptamer (TBA), one of the first aptamers discovered by the SELEX methodology. However, no studies on TBA dealing with the effects of introduction of inversion of polarity sites have been reported yet. In this frame, we have undertaken the synthesis and the study of a mini-library composed of several TBA analogues containing a 3′-3′ or a 5′-5′ inversion of polarity site at different positions into the sequence. Particularly, in this article, we present preliminary results about their structural and biological properties.*

Keywords Thrombin binding aptamer; inversion of polarity site

The mini-library of TBA analogues consists of two series of oligonucleotides, namely ODNs 33 containing 3′-3′ and ODNs 55 containing 5′-5′ inversion of polarity sites. Both series were designed introducing an inversion of polarity site between each residue in the TBA sequence $^{5'}$GGTTGGTGTGGTTGG$^{3'}$. Sequences of the 14 ODNs obtained are listed in Table 1. All oligonucleotides were synthesized by solid phase β-cyanoethyl phosphoramidite chemistry and purified by standard methods and then subjected to a preliminary analysis by ^{1}H NMR and CD spectroscopy. Afterward, the promising sequences were selected for a more detailed analysis. The simple appearance of ^{1}H NMR spectra of ODNs **55 II**, **55 III** and **55 IV** (Figure 1) indicates that, in the conditions used, these modified oligomers form mainly a single well-defined hydrogen-bonded conformation. ^{1}H NMR spectra of the other ODNs showed more than one set of signals suggesting the presence of several species in solution. In some cases a severe line broadening prevents us to obtain information about the number of present species. In order to acquire information about the effects of the inversion of polarity site on the

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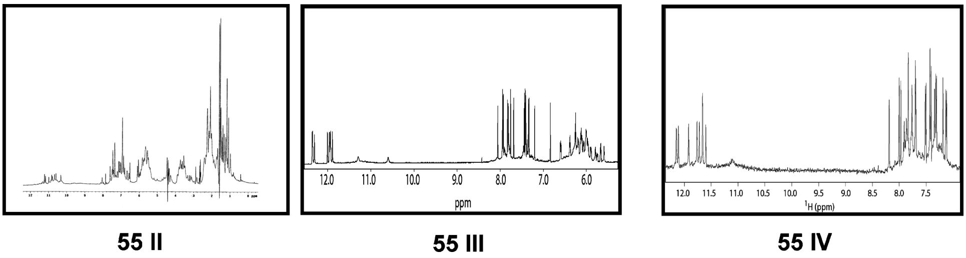
TABLE 1 Sequences of the 14 TBA analogues, containing a 3′-3′ or a 5′-5′ inversion of polarity site at different positions, belonging to the mini-library

5′-5′ Series		3′-3′ Series	
$^{3'}$G$^{5'-5'}$GTTGGTGTGGTTGG$^{3'}$	**55 I**	$^{5'}$G$^{3'-3'}$GTTGGTGTGGTTGG$^{5'}$	**33 I**
$^{3'}$GG$^{5'-5'}$TTGGTGTGGTTGG$^{3'}$	**55 II**	$^{5'}$GG$^{3'-3'}$TTGGTGTGGTTGG$^{5'}$	**33 II**
$^{3'}$GGT$^{5'-5'}$TGGTGTGGTTGG$^{3'}$	**55 III**	$^{5'}$GGT$^{3'-3'}$TGGTGTGGTTGG$^{5'}$	**33 III**
$^{3'}$GGTT$^{5'-5'}$GGTGTGGTTGG$^{3'}$	**55 IV**	$^{5'}$GGTT$^{3'-3'}$GGTGTGGTTGG$^{5'}$	**33 IV**
$^{3'}$GGTTG$^{5'-5'}$GTGTGGTTGG$^{3'}$	**55 V**	$^{5'}$GGTTG$^{3'-3'}$GTGTGGTTGG$^{5'}$	**33 V**
$^{3'}$GGTTGG$^{5'-5'}$TGTGGTTGG$^{3'}$	**55 VI**	$^{5'}$GGTT GG$^{3'-3'}$TGTGGTTGG$^{5'}$	**33 VI**
$^{3'}$GGTTGGT$^{5'-5'}$GTGGTTGG$^{3'}$	**55 VII**	$^{5'}$GGTT GGT$^{3'-3'}$GTGGTTGG$^{5'}$	**33 VII**

structural features of the resulting complexes, CD spectra were acquired for ODNs **55 II**, **55 III**, and **55 IV** and their natural counterpart. All CD spectra exhibit two positive bands around 248 and 294 nm and a negative one around 267 nm. In particular, the CD spectra of **55 III** and TBA, performed at 20°C, are almost superimposable suggesting the formation of an antiparallel quadruplex structure,[1–3] containing residues in *syn*-glycosidic conformations for **55 III**. Melting CD experiments, performed at a scan rate of 10°C/h, provided the melting temperatures of 50°C, 58°C, 61°C, and 54°C for complexes **55 II**, **55 III**, and **55 IV** and TBA, respectively. These results encouraged us to undertake both a more detailed structural investigation and an estimation of anti-thrombin activity of complexes showing an improved thermal stability compared to TBA. Here we mainly report results on the TBA analogue **55 III** ($^{3'}$G1-G2-T3$^{5'-5'}$T4-G5-G6-T7-G8-T9-G10-G11-T12-T13-G14-G15$^{3'}$). The ^{1}H-NMR spectrum of **55 III** (700 MHz, T = 25°C) shows eight well defined signals in the region 11.5–12.5 ppm, attributable to imino protons involved in Hoogsteen hydrogen bonds of at least two G-quartets, and of two broader and less intense singlets at 10.6 and 11.29 ppm, respectively, probably belonging to two imino protons of two thymines involved in mutual hydrogen bonding. Moreover, 15 signals in the aromatic region due to the presence of nine guanine H8 and six thymine H6 protons were clearly observable. A combination of the analysis of 2D NOESY (700 MHz, T = 25°C), TOCSY spectra (700 MHz, T = 25°C), and ^{31}P-NMR



55 II **55 III** **55 IV**

FIGURE 1 ^{1}H NMR spectra of **55 II**, **55 III**, and **55 IV**.

TABLE 2 Proton chemical shifts (700 MHz) of **55 III** in 10 mM KH_2PO_4, 70 mM KCl, 0.2 mM EDTA (pH 7.0, T = 25°C)

Base	H8/H6	H1′	H2′	H2″	H3′	H4′	H5′[a]	H5″[a]	H2/Me	NH′[b]	^{31}P
3′G1	7.94	6.15	2.39	2.56	4.73	4.18	4.21	3.94		11.91	−1.72
G2	6.84	5.68	3.77	2.94	4.83	4.42	4.23	4.27		12.35	−1.51
5′T3	7.83	6.61	2.31	2.45	4.92	4.41	3.69	4.01	1.98		−1.60
5′T4	7.36	5.76	1.80	2.14	4.56	4.45	3.84		1.37	11.35	−1.36
G5	7.42	6.01	3.41	2.92	4.90	4.30	4.39			12.30	−0.89
G6	7.70	5.90	2.58	2.71	5.10	4.40	4.21	3.95		12.03	−1.09
T7	7.85	6.40	2.44	2.58	4.84	4.41	4.22	3.76	1.97		−1.56
G8	7.43	5.68	2.02	2.33	4.69	4.13	4.07	3.81			−1.76
T9	7.22	5.80	1.90	2.31	4.59	4.48	3.67	3.51	1.54		−1.98
G10	7.45	6.02	3.64	2.90	4.90	4.37	4.15	4.34		11.91	−1.84
G11	8.07	5.98	2.32	2.90	5.09	4.40	4.25			12.37	−0.53
T12	7.77	6.26	2.27	2.59	4.90	4.35	4.24	4.34	1.96		−2.20
T13	7.34	6.20	2.19	2.76	4.93	4.30	4.24	4.33	1.35	10.65	−1.01
G14	7.41	6.09	3.63	3.00	4.96	4.58	4.30	4.38		11.95	−1.80
3′G15	7.96	6.12	2.39	2.59	4.82	4.53	4.20	4.30		11.97	

[a] No stereospecific assignment has been done for H5′ and H5″.

[b] Imino proton chemical shifts have been assigned at T = 5°C.

spectroscopy (202 MHz, T = 25 °C) allowed us to get the almost complete assignment (Table 2) of both exchangeable and nonexchangeable protons and phosphorus resonances of **55 III**. Interestingly, the intensities of NOESY (700 MHz, T = 25 °C, mixing time 100 ms) crosspeaks between the H8 proton bases and sugar H1′ resonances indicate that four (G2, G5, G10, G14) out of nine Gs of **55 III** adopt a *syn* glycosidic conformation, (Figure 2) where the H8 resonances of *syn* G residues are upfield shifted with respect to those of the *anti* ones.[4–6] Then, four *anti*-Gs (G1, G6, G11, and G15) have classical H8/H2′-H2″ sequential connectivities to 5′ neighboring *syn*-Gs (G2, G5, G10 and G14, respectively) indicating that the subunits G1-G2, G5-G6, G10-G11, and G14-G15 are involved in the formation of a four-stranded helical structure (underlined residues adopt a *syn* glycosidic conformation). The alternation of *syn* and *anti* G residues within each strand suggests that, as TBA, **55 III** folds into a monomolecular foldback quadruplex,

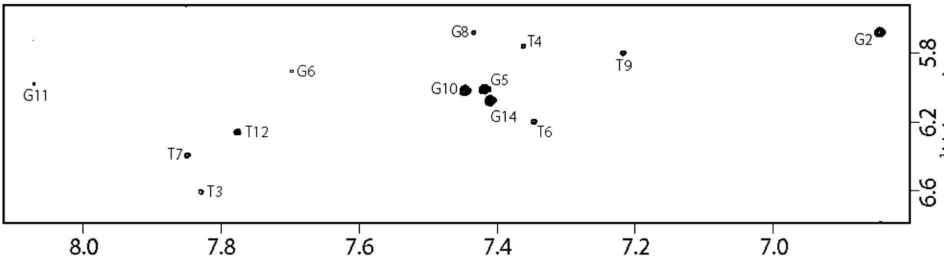
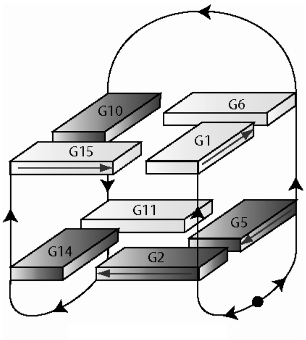


FIGURE 2 Expanded region of the NOESY spectrum of **55 III** (700 MHz, T = 25°C, mt = 200 ms) correlating G-H8 and H1′ protons.



55 III

FIGURE 3 Schematic illustration of the structure adopted by **55 III**.

characterized by two G tetrads. Further, a number of unusual NOE connectivities was observed between *syn*-Gs and Ts, indicating that ${}^{5'}TG^{3'}$ and ${}^{5'}GT^{3'}$ tracts do not adopt a helical winding, thus suggesting that, most probably, the TT and TGT tracts form loops. The acquisition of a ${}^{1}H$-${}^{15}N$ HSQC experiment allowed us to assign exchangeable protons to their respective G or T residues. To be noted that signals at 10.65 and 11.35 ppm belonging to T residues are characterized by strong NOE effects between each other, thus suggesting that two thymines are in close proximity and interacting by a mutual hydrogen bond, likewise as observed in the unmodified TBA. The whole of data suggests that the structure of **55 III** is a foldback quadruplex characterized by two G tetrads formed by the residues G1, G6, G10, G15 and G2, G5, G11, G14, respectively, where the underlined residues possess a *syn* glycosidic conformation. Interestingly the two tetrads assume an *anti-anti-anti-syn* and *syn-syn-syn-anti* arrangement of the bases. Hence, **55 III** folds into a chair-like quadruplex structure possessing three strands parallel to each other and only one strand oriented in the opposite direction (Figure 3). In order to study the effects of the structural changes on the biological activity of **55 III**, if any, we performed a prothrombin time (PT) assay. PT assay on **55 III** was performed on human plasma in strict comparison with TBA. In order to eliminate the variation caused by the measurements performed on different days, the samples of **55 III** and TBA were prepared at the same time and folded together by heating the samples for 10 minutes at 80°C and slowly cooling them down at room temperature. The assays have been conducted after one week from the preparation procedure. As shown in Figure 4, overall, TBA showed a statistically significant more marked effect of **55 III** both as onset of the activity and endurance. Indeed, incubation studies demonstrated that TBA effect is already present after 10 seconds of incubation and lasts up to 15 minutes of incubation. On the other hand, **55 III** showed a slower onset of the effect, indeed PT time started to be significantly prolonged following 5 minutes of incubation. Also the magnitude

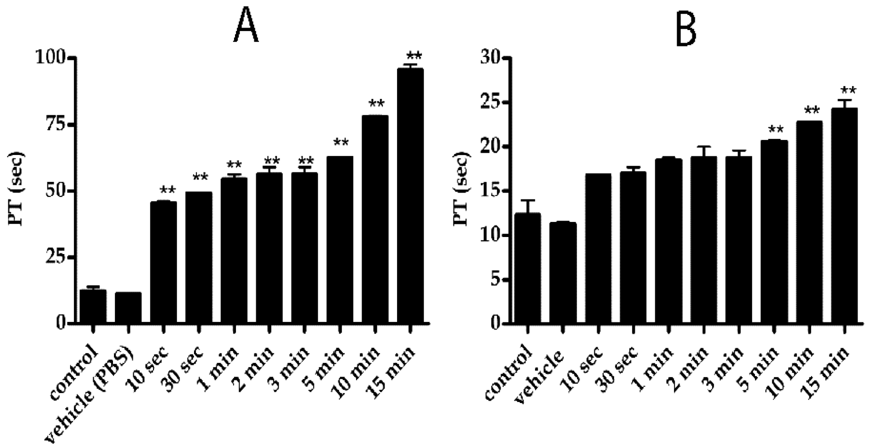


FIGURE 4 Prothrombin time assessed by using human plasma. On the x-axis the minutes of incubation are reported, while PT time in seconds is reported on y-axis. Panel A refers to TBA, panel B to **55 III**.

of the activity of **55 III** resulted reduced compared to TBA. The biological data reported here imply that the modifications of the structure adopted by **55 III** affect sensibly the biological activity, and therefore its interaction with the thrombin.

The ODNs of the mini-library show quite different structural behavior. There is evidence for a main stable conformation only for the 5′-5′ series (ODNs **55 II**, **55 III**, and **55 IV**). In fact, most of ^{1}H NMR spectra of ODNs belonging to the 3′-3′ series showed more than one set of signals suggesting the presence of several species in solution. Furthermore, an inversion of polarity site 5′-5′ positioned in the G-tract prevents the formation of a stable quadruplex structure. However, it could be of interest investigate the different conformations present in solution, since, in some cases (for example ODN **55 VI**) a significant anticoagulant activity resulted, in spite of its rather ambiguous ^{1}H NMR spectrum (data not shown).

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