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Supporting Information

Chemical Maturation of a Bivalent Aptamer by Single Domain Variation

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[a]Life and Medical Sciences Institute, University of Bonn, Gerhard-Domagk-Str. 1, 53121 Bonn, Germany, [b]Nucleic Acid Center, Department of Physics, Chemistry and Pharmacy, University of Southern Denmark, Campusvej 55, 5230 Odense M, Denmark, [c]Institute for Experimental Hematology and Transfusion Medicine, University Hospital Bonn, Sigmund Freud Str. 25, 53127 Bonn, Germany

Experimental section

Filter retention assay

The affinities of the aptamers used in this study to their target molecule thrombin were analyzed by filter retention. 10 pmol aptamer were radioactive 5´ phosphorylated by 20 U T4 Polynucleotide Kinase (New England BioLabs) in 70 mM Tris-HCl buffer pH 7.6 containing 10 mM $MgCl_2$, 5 mM Dithiothreitol and 300 µM γ-32P-ATP. γ-32P-ATP was purchased from PerkinElmer. Different Thrombin concentrations were incubated with 0.5 nM 32P-labeled aptamer 30 min at 37 °C in PBS pH 7.4 containing 1 mg/ml BSA, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Thrombin was purchased from Haematologic Technologies Inc. After incubation, the reactions were passed through 0.45 µm nitrocellulose membranes (Whatman) and washed 3 times with 200 µl PBS buffer containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Bound aptamers were quantified with the Fujifilm FLA 3000 PhosphorImager.

CD-spectroscopy

CD spectra were recorded by a Jasco J-810 circular dichroism spectropolarimeter using a quartz cell with 1 mm optical path length and a wavelength range from 230 to 320 nm at 1 nm bandwidth, 1 nm step size, and 0.5 s time per point. 50 µM final aptamer concentration in PBS pH 7.4 were heated to 95 °C for 1 min and cooled to room temperature for 30 min. The CD spectra were obtained by taking the average of seven scans.

Melting temperature

The melting temperature (T_M) was determined by a Perkin-Elmer UV/Vis Spectrometer Lambda 2S with a Peltier-controlled thermostatting PTP-1 system using a quartz cell with 1 cm optical path length. Absorbance of 10 µM final aptamer concentration in PBS pH 7.4 was measured at 295 nm as a function of temperature. Absorbance versus temperature curves from 25 to 90 °C with a heating rate of 0.5 °C/min were differentiated to determine T_M values.

Thrombin time

The anticoagulant activity of aptamer variants was analyzed by a one-stage plasma-based thrombin-time (TT) assay using an Amelung KC10 A Coagulometer (Amelung, Lemgo, Germany). Human α-thrombin (CellSystems, Troisdorf, Germany) was diluted in assay buffer (1 ×PBS, pH 7.4, 3 mM $MgCl_2$, 1 µg/µl BSA) to reach a final concentration of 10 NIH U/ml (75 nM). This thrombin solution was spiked with the aptamer variants to yield the indicated concentrations. After incubation of 75 µL-aliquots of this solution for 4 min at 37°C in measuring cuvettes, 75 µl of normal pooled human plasma (NPP) were added and

the formation time of a fibrin clot, resulting from direct cleavage of plasma fibrinogen by thrombin, measured.

Activated partial thromboplastin time

Aptamer variants were added to NPP at indicated concentrations and samples introduced to activated partial thromboplastin time (aPTT) measurements. In clinical diagnostics, this plasma-based two-stage clotting assay is used to assess the overall coagulation potency in patient plasma or for the monitoring of anticoagulant therapies by e.g. thrombin-inhibiting drugs. Analysis was done using an automated BCS XP coagulation analyzer (Siemens Healthcare Diagnostics, Marburg, Germany). During the process, 50 µl of plasma samples were added to measuring cuvettes and 50 µl of Actin FS solution (Siemens) added. This reagent contains ellagic acid to activate the so-called contact phase of the plasmatic coagulation cascade. After incubation at 37°C for 3 min, to allow for Ca^{2+}-dependent generation of thrombin and following clot formation, 50 µl of a $CaCl_2$-solution (25 mM) were added and clotting times measured.

Whole blood clotting assay (ROTEM®)

Rotation thrombelastometry (ROTEM®) was used to assess the anticoagulant activity of aptamers and bivalirudin in whole blood. At this, a ROTEM® coagulation analyzer (TEM International, Munich, Germany) was used to measure the whole blood clotting time (CT). In brief, citrated whole blood was aliquoted and spiked with molecules to yield final concentrations of 1 µmol/L each. Subsequently, 320 µl of each sample and 20 µl of starting solution (0.1 M Hepes, pH 7.4; 0.2 M $CaCl_2$) were added to fixed cuvettes, mixed, and clotting times (initial change of viscosity) automatically measured by the ROTEM® system. AD1-22 sequence: 5′-ACCACGGACTTTTTTTTTTTTTTTCCAACCACAC-3′

Oligonucleotide synthesis and purification

Oligonucleotide synthesis was carried out on an Expedite™ Nucleic Acid Synthesis System Model 8909 from Applied Biosystems in 200 nmol scale on 500 Å CPG supports with 1H-tetrazole as an activator for coupling reaction. The amidites **P**, **O** and **Y** were dissolved in dry dichloromethane and inserted into the growing oligonucleotide chain using an extended coupling time (15 min). In case of HD1-22 modified aptamers, the synthesis was carried out in 1000 nmol scale. Coupling efficiencies for monomers **P**, **O** and **Y** as well as the overall yield were shown in Table S1 based on the difference in absorbance of the dimethoxytrityl cation released. Cleavage from solid support and removal of nucleobase protecting groups was performed using standard conditions (32% aqueous ammonia for overnight at 55 ºC). The modified oligonucleotide aptamers were purified by DMT-off RP-HPLC program using Waters system 600 in which the XBridge precolumn (10 × 10 mm, 5µm) was equipped with XBridge OST C18(10 × 50 mm, 2.5µm) Chromatography System. Elution was performed starting with an isocratic hold of buffer A for 2 min followed by a linear gradient to 70% buffer B in 17 min then linear gradient to 100% buffer B in 0.5 min and then 100% buffer A in 5.2 min at a flow rate of 2.5 ml /min at oven temperature 45°C (Buffer A: 0.05M triethylammonium acetate (pH 7.4); Buffer B: 75% MeCN/25% Buffer A). Precipitation was accomplished using pure acetone via addition of 100 µl double filtered water followed by aqueous sodium perchlorate (5 M, 15 µl) then pure acetone (1 ml). HD1 type modified ODNs were confirmed by MALDI-TOF analysis on a Ultraflex II TOF/TOF system from Bruker (a MALDI-LIFT system) with HPA-matrix (10 mg 3-hydroxypicolinic acid, in 50 mM ammoniumcitrate/70% acetonitril) matrix as shown in Table S1. The purity of the final TFOs was found to be over 95%, checked by ion-exchange chromatography using La-Chrom system from Merck Hitachi on Dionex DNAPac Pa-100, 4 ×250 mm Analytical column using perchlorate buffer at pH 8 with 1 ml /min flow rate.

NMR experiments

The TINA-modified molecule, HD1 r8P, was dissolved in 0.5 mL mixture of H_2O and D_2O (9:1) with 10 mM sodium phosphate buffer (pH 7.0) and 100 mM KCl. The concentration of the oligonucleotide was 0.7 mM. NMR experiments were performed on a Varian UNITY 500 spectrometer at 25°C. A NOESY

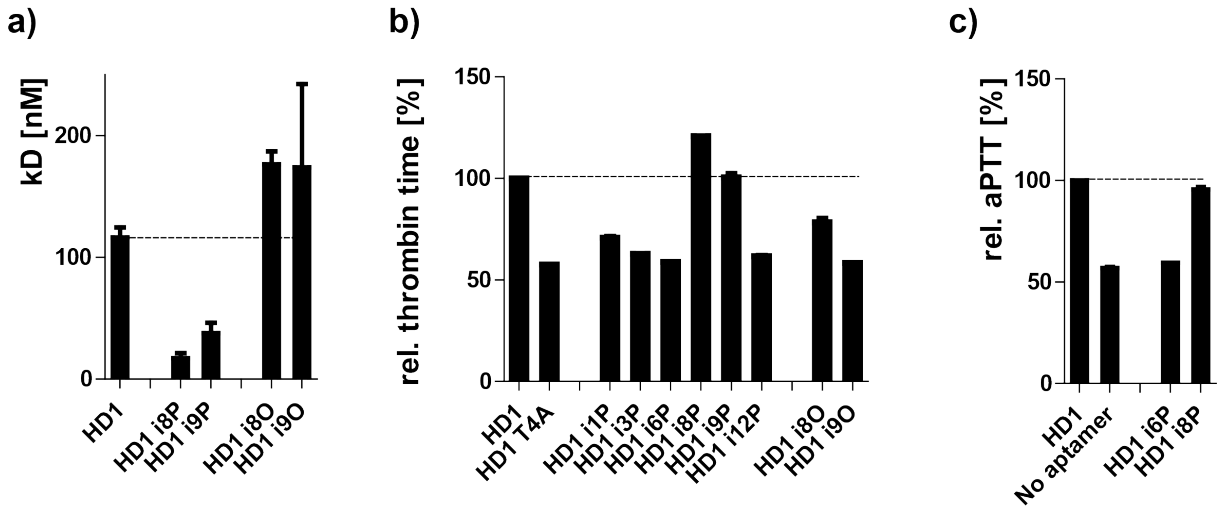
spectrum with a mixing time of 250 ms was acquired using the WATERGATE pulse sequence with 2048 complex points in t_2 and a spectral width of 10,000 Hz. A total of 512 t_1 experiments, each with 120 scans and a dwell-time of 2.0 s between scans, were recorded using the States phase cycling scheme.

Supporting Table S1

Oligonucleotide	Sequence	Overall yield (%)	**P**,**O** and **Y** coupling (%)	Found *m/z* [M-H]$^-$	Calc. *m/z* [M-H]$^-$
HD1 r7P	GGTTGGPGTGGTTGG	82	96	4887.0	4889.2
HD1 r8P	GGTTGGTPTGGTTGG	78	94	4862.0	4864.2
HD1 r9P	GGTTGGTGPGGTTGG	82	95	4885.9	4889.2
HD1 r7O	GGTTGGOGTGGTTGG	73	90	4888.9	4889.8
HD1 r8O	GGTTGGTOTGGTTGG	70	92	4864.5	4864.8
HD1 r9O	GGTTGGTGOGGTTGG	78	92	4895.9	4889.8
HD1 r7O r8P	GGTTGGOPTGGTTGG	78	97 (P) 94 (O)	5028.6	5028.0
HD1 r7Y	GGTTGGYGTGGTTGG	82	100	4881.4	4880.8
HD1 r8Y	GGTTGGTYTGGTTGG	81	99	4858.4	4855.8
HD1 r9Y	GGTTGGTGYGGTTGG	81	100	4881.8	4880.8
HD1 i1P	PGGTTGGTGTGGTTGG	78	-	5196.5	5193.4
HD1 i3P	GGPTTGGTGTGGTTGG	76	92	5193.9	5193.4
HD1 i6P	GGTTGPGTGTGGTTGG	78	95	5190.1	5193.4
HD1 i8P	GGTTGGPTGTGGTTGG	79	96	5197.4	5193.4
HD1 i9P	GGTTGGTPGTGGTTGG	79	97	5195.6	5193.4
HD1 i12P	GGTTGGTGTGGPTTGG	76	94	5196.5	5193.4
HD1 i8O	GGTTGGTOGTGGTTGG	76	89	5190.7	5194.0
HD1 i9O	GGTTGGTGOTGGTTGG	75	91	5193.2	5194.0
HD1-22 r8P	GGTTGGTPTGGTTGGAAAAAAAAAAAAAAA AGTCCGTGGTAGGGCAGGTTGGGGTGACT	86	93	[a]	18710.0
HD1-22 r7O	GGTTGGOGTGGTTGGAAAAAAAAAAAAAA AAGTCCGTGGTAGGGCAGGTTGGGGTGACT	78	86	[a]	18735.6
HD1-22 r8Y	GGTTGGTYTGGTTGGAAAAAAAAAAAAAA AAGTCCGTGGTAGGGCAGGTTGGGGTGACT	90	98	[a]	18701.6

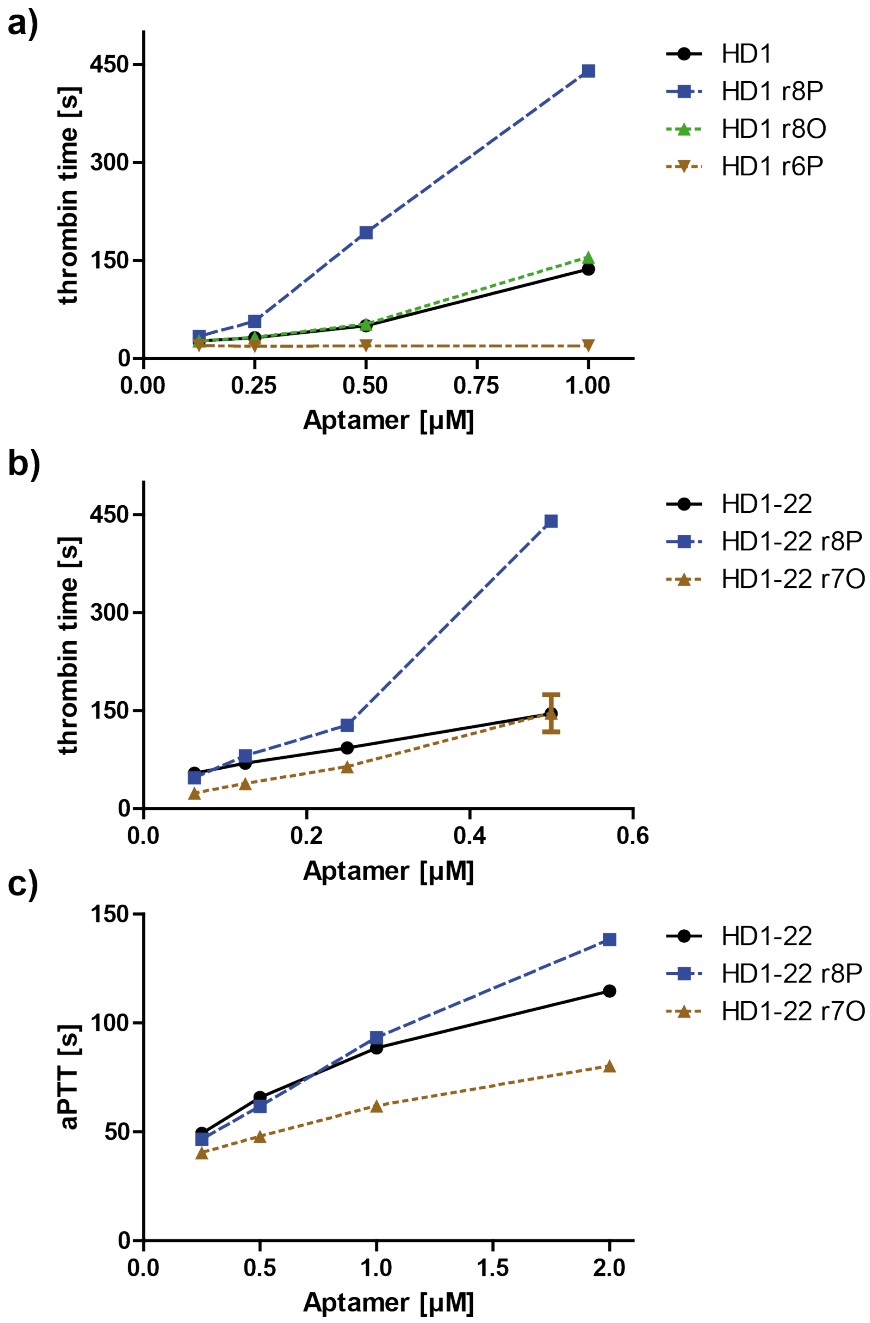
[a]MALDI-TOF analysis on a Ultraflex II TOF/TOF system from Bruker not possible.

Supporting figure 1



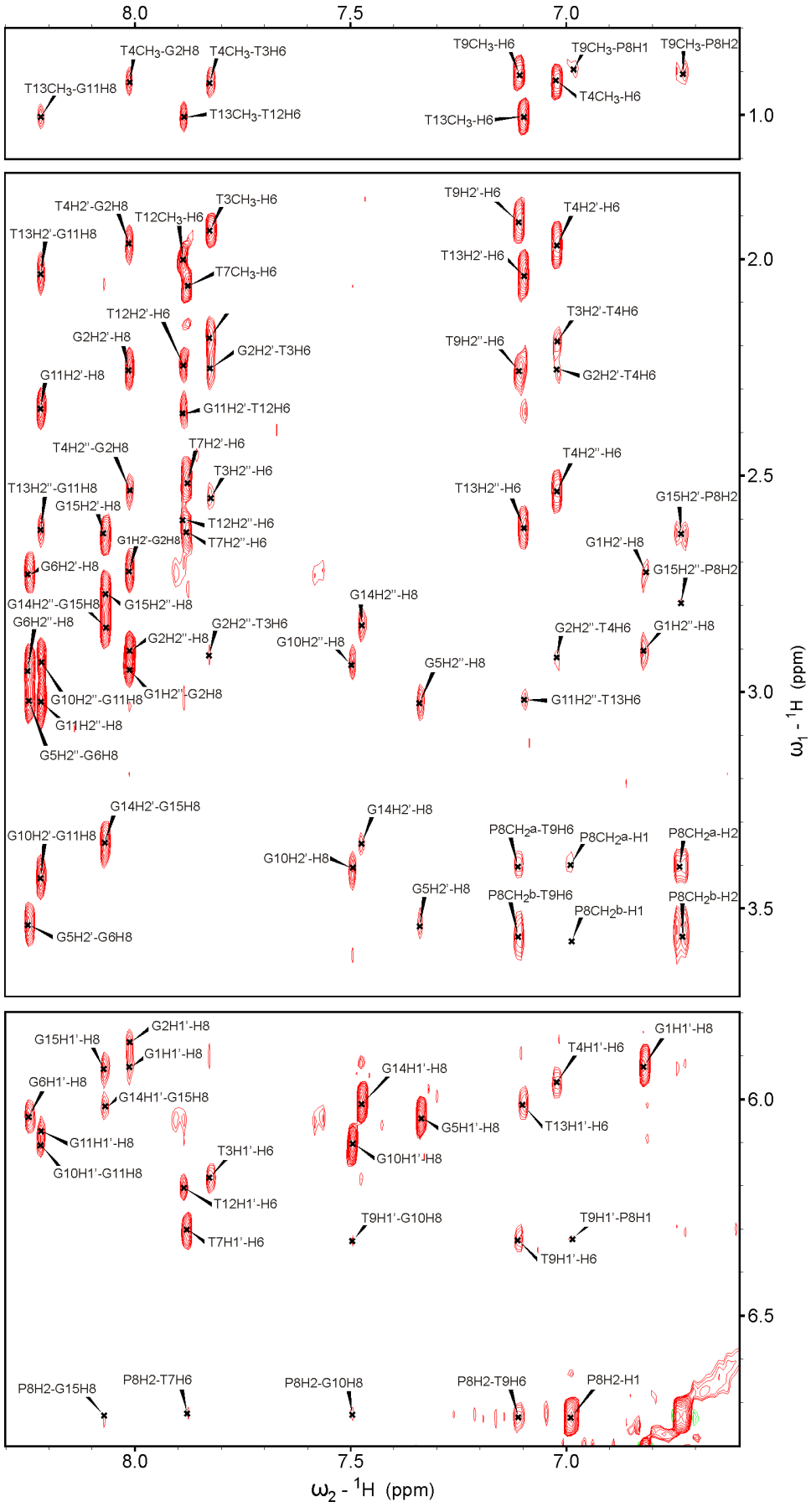
Supporting Figure 1: a) Dissociation constants, b) thrombin time and c) activated partial thromboplastine time (aPTT) of PAH-aptamer insertion variants in relation to HD1. HD1 T4A: non-binding single mutation control oligonucleotide.

Supporting figure 2



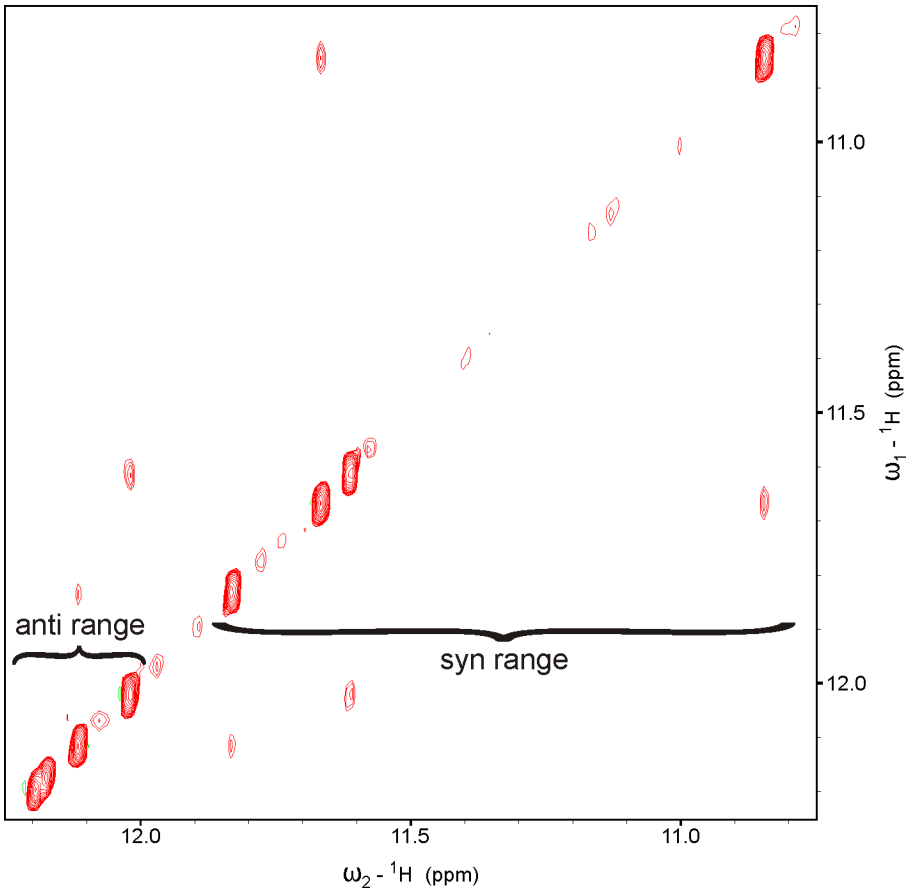
Supporting Figure 2: Concentration dependent anticoagulant activity of selected PAH-modified variants as measured by a) and b) thrombin time and c) activated partial thromboplastine time (aPTT).

Supporting figure 3



Supporting Figure 3: Excerpts of a 250 ms NOESY spectrum of HD1 r8P recorded at 25°C in 9:1 H_2O:D_2O (pH = 7, 100 mM K^+). Shown is the aromatic–methyl and H2'/H2'' region (top two panels) and the aromatic–H1' region (bottom panel). Assignments are indicated for cross peaks.

Supporting figure 4



Supporting Figure 4: The imino–imino region of a 250 ms NOESY spectrum of HD1 r8P recorded at 25°C in 9:1 H_2O:D_2O (pH = 7, 100 mM K^+). Four resonances are observed in the typical range for anti guanosines and four in the range for syn residues. The imino resonances are not assigned.