ELECTRONIC SUPPLEMENTARY INFORMATION

A straightforward modification in the thrombin binding aptamer improving stability, affinity to thrombin and nuclease resistance

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EXPERIMENTAL SECTION

Oligonucleotides synthesis and purification

The oligonucleotides in **Table 1** were synthesized on a Millipore Cyclone Plus DNA synthesizer using solid phase β-cyanoethyl phosphoramidite chemistry at 15 μmol scale. The synthesis of the 3'-5' tracts were performed by using normal 3'-phosphoramidites, whereas the 5'-3' tracts were synthesized by using 5'-phosphoramidites. For all ODNs an universal support was used.

The oligomers were detached from the support and deprotected by treatment with concentrated aqueous ammonia at 80°C overnight. The combined filtrates and washings were concentrated under reduced pressure, redissolved in H_2O, analyzed and purified by high-performance liquid chromatography on a Nucleogel SAX column (Macherey–Nagel, 1000-8/46), using buffer A: 20 mM NaH_2PO_4/Na_2HPO_4 aqueous solution (pH 7.0) containing 20% (v/v) CH_3CN and buffer B: 1 M NaCl, 20 mM NaH_2PO_4/Na_2HPO_4 aqueous solution (pH 7.0) containing 20% (v/v) CH_3CN; a linear gradient from 0 to 100% B for 45 min and flow rate 1 ml/min were used. The fractions of the oligomers were collected and successively desalted by Sep-pak cartridges (C-18). The isolated oligomers proved to be >98% pure by NMR (700 MHz, D_2O, 50°C) (Fig. S4 and S5) and HPLC (Macherey–Nagel, 1000-8/46, buffer A: 20 mM NaH_2PO_4/Na_2HPO_4 aqueous solution (pH 7.0) containing 20% (v/v) CH_3CN; buffer B: 1 M NaCl, 20 mM NaH_2PO_4/Na_2HPO_4 aqueous solution (pH 7.0) containing 20% (v/v) CH_3CN; linear gradient from 0 to 100% B for 30 min and flow rate 1 ml/min) (Fig. S6-S9).

CD spectroscopy

CD samples of modified oligonucleotides and their natural counterpart were prepared at a ODN concentration of 100 μM by using PBS (Sigma-Aldrich; 10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4) and submitted to the annealing procedure (heating at 90°C and slowly cooling at room temperature). CD spectra of all quadruplexes and CD melting/annealing curves were registered on a Jasco 715 CD spectrophotometer. For the CD spectra, the wavelength was varied from 220 to 320 nm at 100 nm min^{-1} scan rate, and the spectra recorded with a response of 16 s, at 2.0 nm bandwidth and normalized by subtraction of the background scan with buffer. The temperature was kept constant at 20°C with a thermoelectrically-controlled cell holder (Jasco PTC-348). CD melting curves were registered as a function of temperature (range: 20°-90°C) for all quadruplexes at their maximum Cotton effect wavelengths. The CD data were recorded in a 0.1 cm pathlength cuvette with a scan rate of 0.5°C/min.

UV thermal difference spectra (TDS)

UV samples of investigated oligonucleotides were prepared using a buffer solution: lithium cacodylate (10 mM, pH 7.4), NaCl (137 mM). For each oligonucleotide sample, a UV spectrum was recorded above and below its melting temperature (T_m). All experiments were performed on a Jasco V 530 UV/Vis spectrophotometer using quartz cuvettes with an optical path of 1 cm and at 40 μM strand concentration. Absorbance spectra were recorded in the 220-320 nm range, with a scan speed of 200 nm min^{-1} and with a data interval of 1 nm. The difference between the UV spectra at high (90°C) and low (20°C) temperatures was defined as the TDS; this represents the spectral difference between the unfolded and folded forms. The temperature (20 or 90°C) was kept constant with a thermoelectrically controlled cell holder (Jasco PTC-348). The thermal difference spectra were normalized (+1 for the highest positive peak).

Fibrinogen Assay

The fibrinogen clotting times were measured spectrophotometrically. ODNs were incubated for 1 min at 37°C in 1.0 mL of PBS containing 2.0 mg/mL of fibrinogen (Fibrinogen from human plasma, F 3879, Sigma-Aldrich) in a PMMA cuvette (vol. 1.5 mL, c.o. 1 cm, Brand). 100 μL of human thrombin (10 NIH per mL; Sigma-Aldrich, T8885, human thrombin suitable for thrombin

time test) was then added to the solution containing the fibrinogen and the ODN. The time required to fibrin polymerization was determined from UV scattering curve, registered as a function of the time (wavelength fixed at 380 nm) in the presence of each ODN. Each curve was determined in triplicate at different concentrations. The clotting time value reported as M ± SE, was derived as the maximum of the second derivative of each scattering curve. The basal clotting time was determined by measuring the UV scattering as a function of the time produced in the absence of any ODN. In the absence of any inhibitor, the clotting time value was 12.0 ±1.5 s. The prolonged fibrinogen clotting times of TBA and modified ODNs were calculated by subtracting the basal clotting time produced by thrombin from that measured in the presence of the aptamer.

Nuclease stability assay

Nuclease stability of anti-thrombin aptamers was conducted in 10% fetal bovine serum (FBS, Gibco) diluted with Dulbecco's Modified Eagle's Medium (DMEM, Lonza) at 37°C. Approximately 8 mmol of stock solution of each ODN (3 O.D.U) was evaporated to dryness under reduced pressure and then incubated with 300 µl 10% FBS at 37°C. At 0, 0.5, 1, 2, 6 and 24 h, 50 µl of samples were collected and stored at –20°C for at least 20 min. The samples were evaporated to dryness and then resuspended in 10 µl of gel loading buffer (30% glycerol, 30% formamide, 40% TE buffer 1x (Tris HCl 1M pH 7,5, EDTA 0,5 M pH 8.0) and 10 µl of autoclaved water. The mixture was used for denaturing polyacrylamide gel electrophoresis (Urea PAGE), carried out at room temperature using 20% polyacrylamide gel in 0.5 x TBE buffer (Tris-borate-EDTA) and 7 M urea. The degradation patterns on the gels were visualized by UV shadowing.

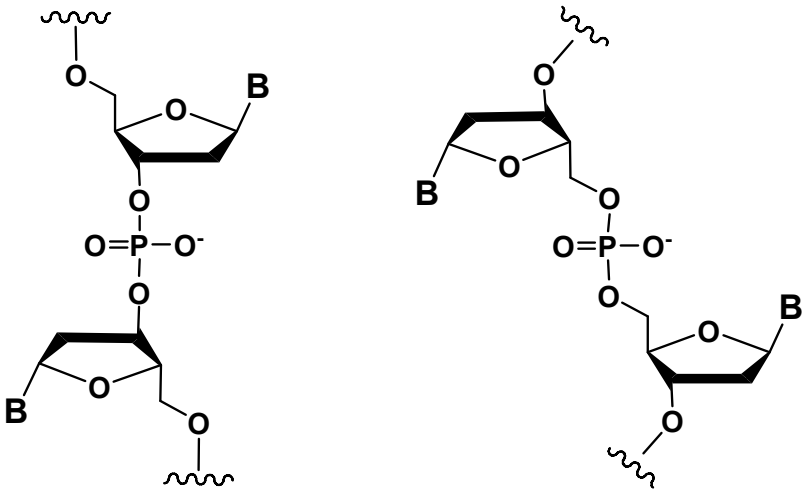


Figure S1. Structures of inversion of polarity sites (IPS) 3'-3' and 5'-5'. **B** = base.

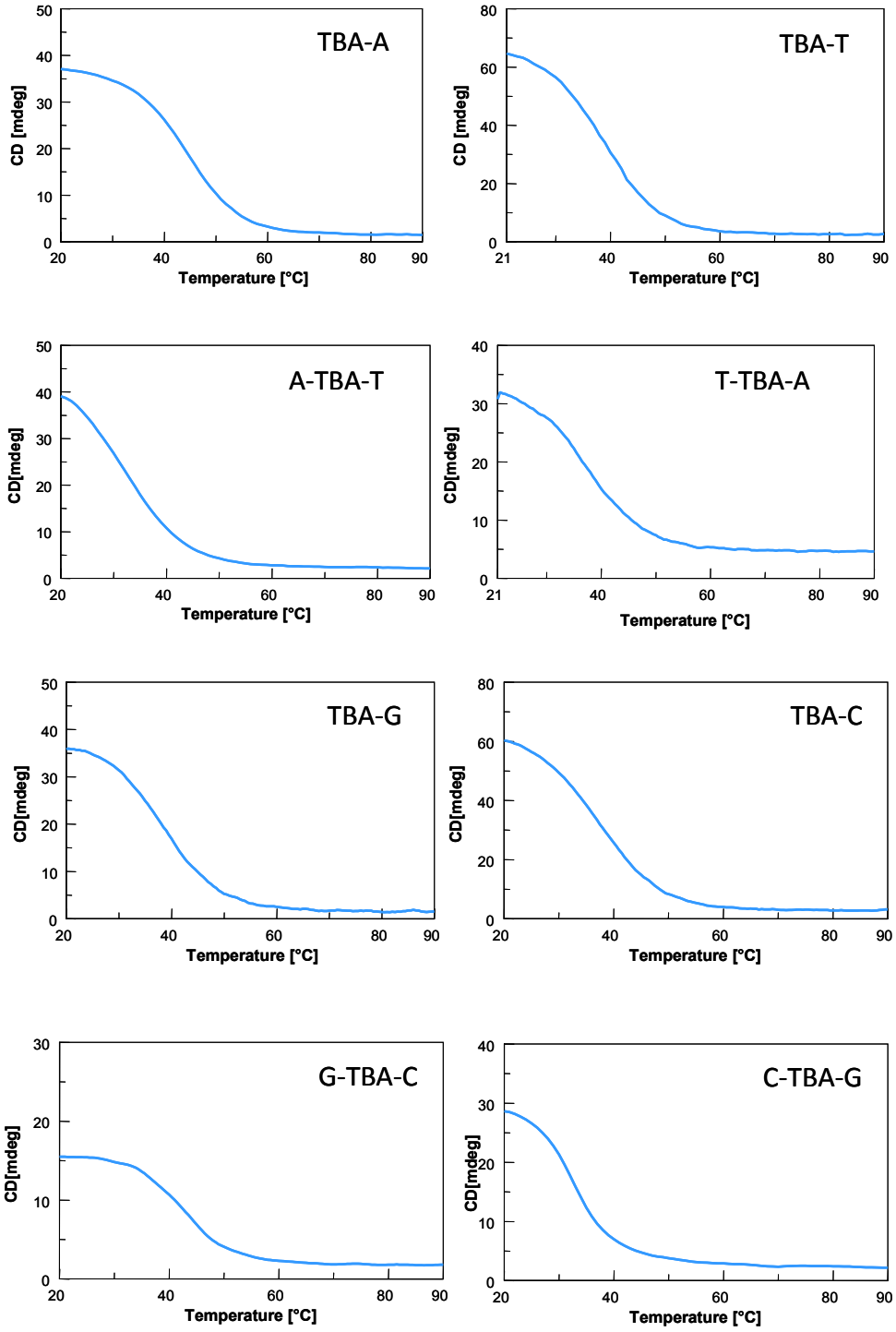


Figure S2. CD melting curves of modified TBAs registered as a function of temperature from 20°C to 90°C for all quadruplexes at their maximum Cotton effect wavelengths (292-295 nm). The CD data were recorded in a 0.1 cm pathlength cuvette at 100 μM ODN strand concentration in PBS, with a scan rate of 0.5°C/min.

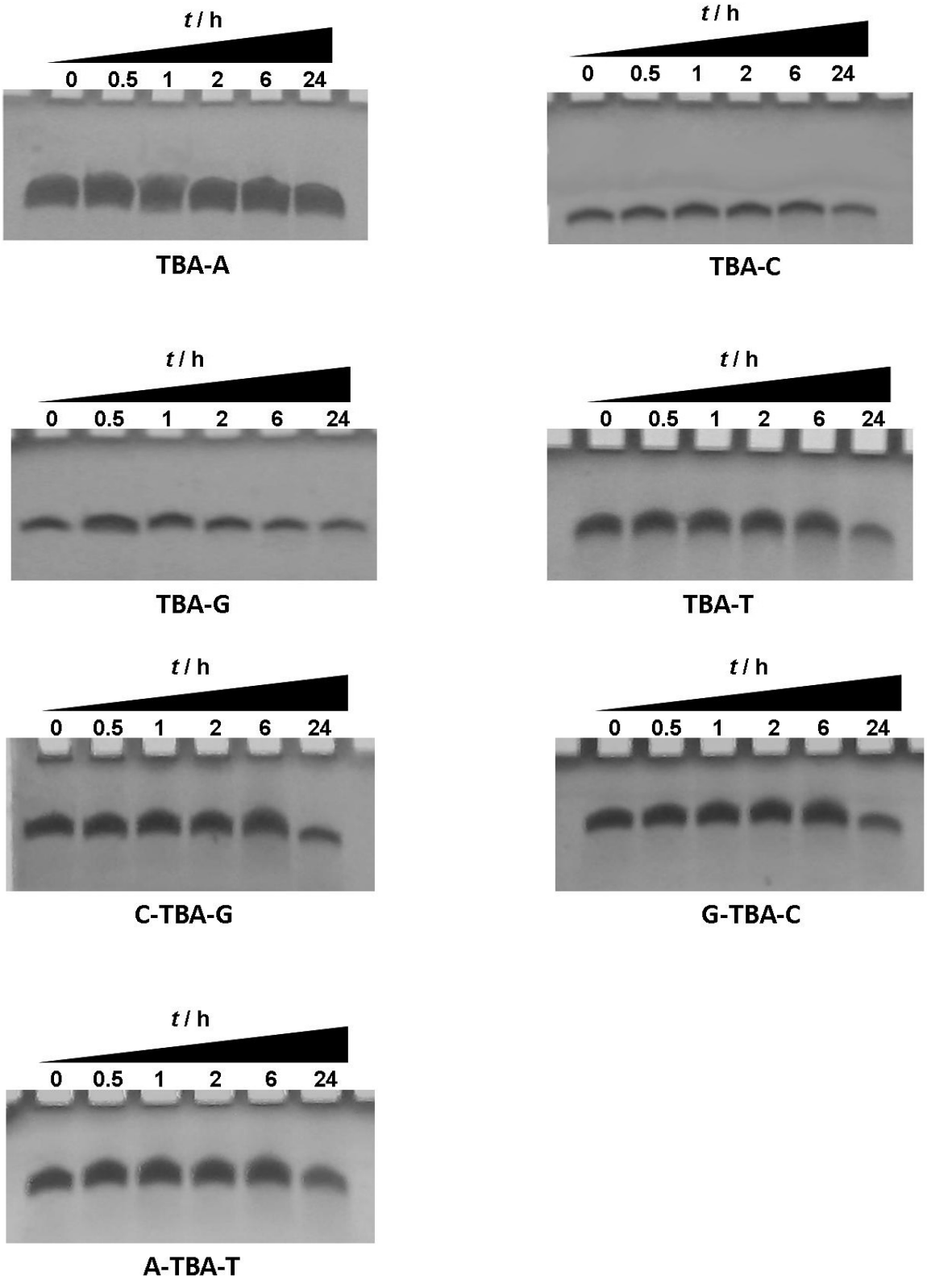


Figure S3. Stability of the modified TBAs in 10% foetal bovine serum (FBS), as monitored by denaturing PAGE (time points h, are shown in the figure).

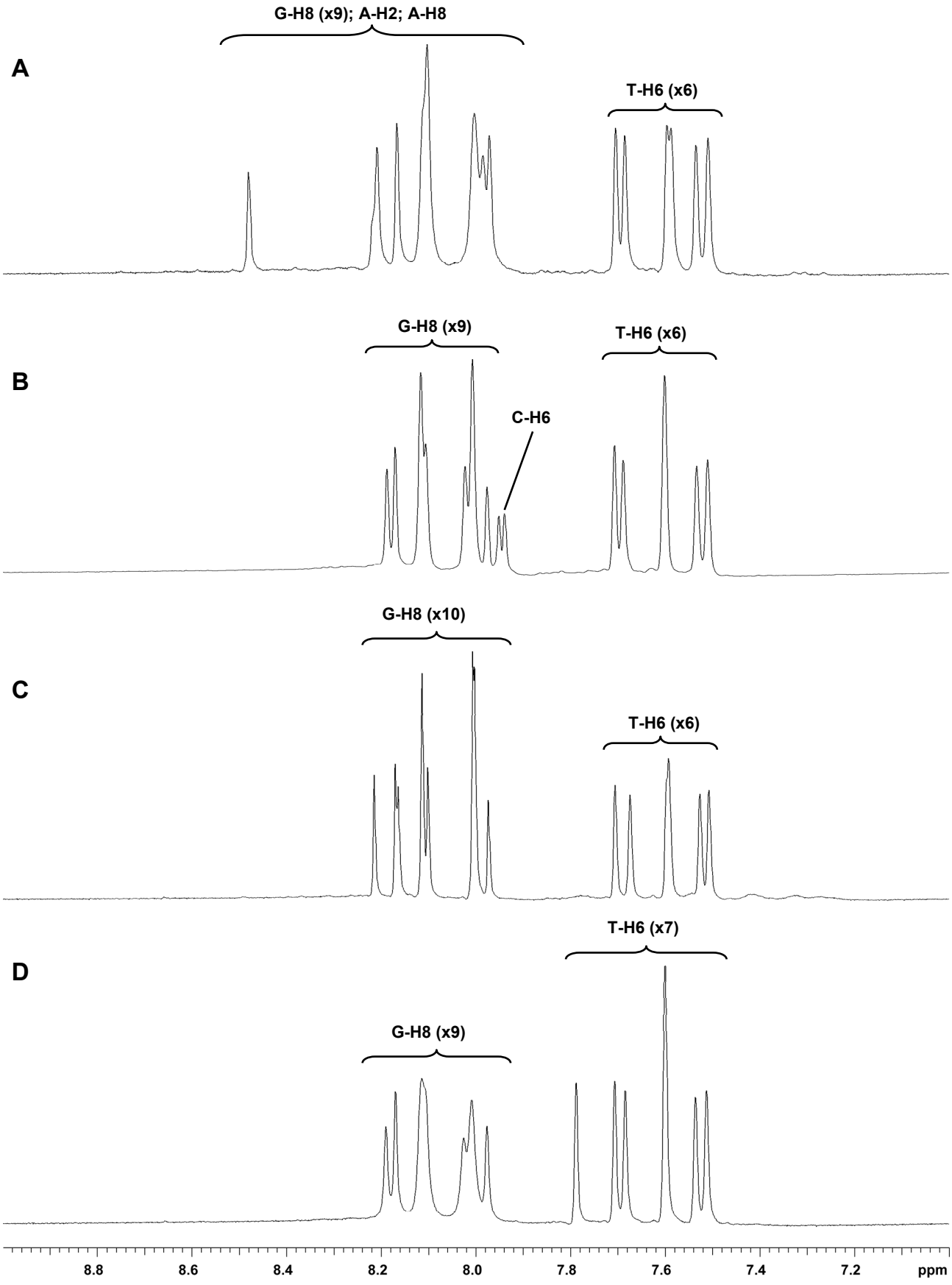


Figure S4. Aromatic region of the high resolution NMR spectra (700 MHz, D_2O, 50°C, no salt) of 5'-GGTTGGTGTGGTTGG-3'-3'-A (**A**), 5'-GGTTGGTGTGGTTGG-3'-3'-C (**B**), 5'-GGTTGGTGTGGTTGG-3'-3'-G (**C**) and 5'-GGTTGGTGTGGTTGG-3'-3'-T (**D**).

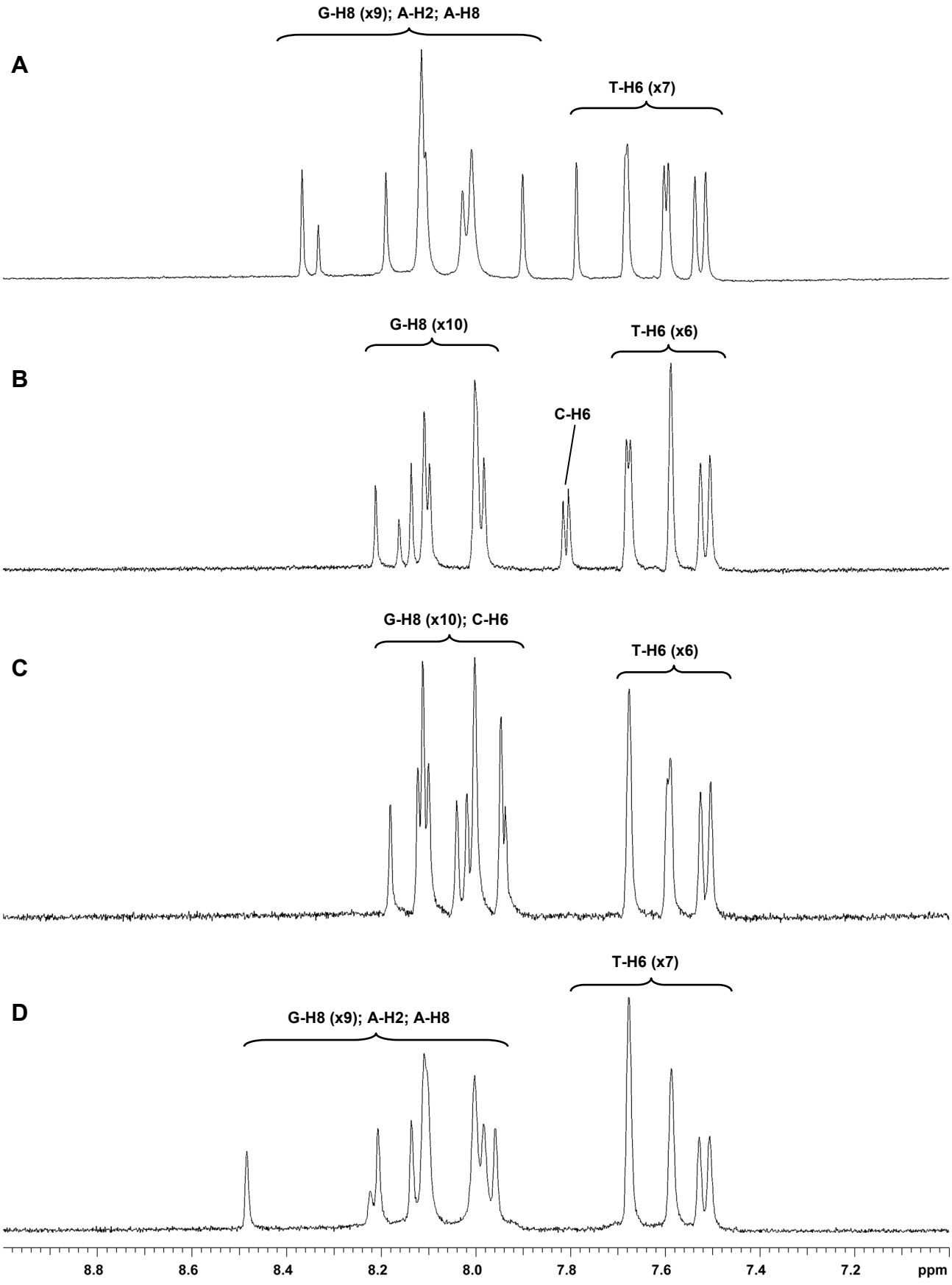
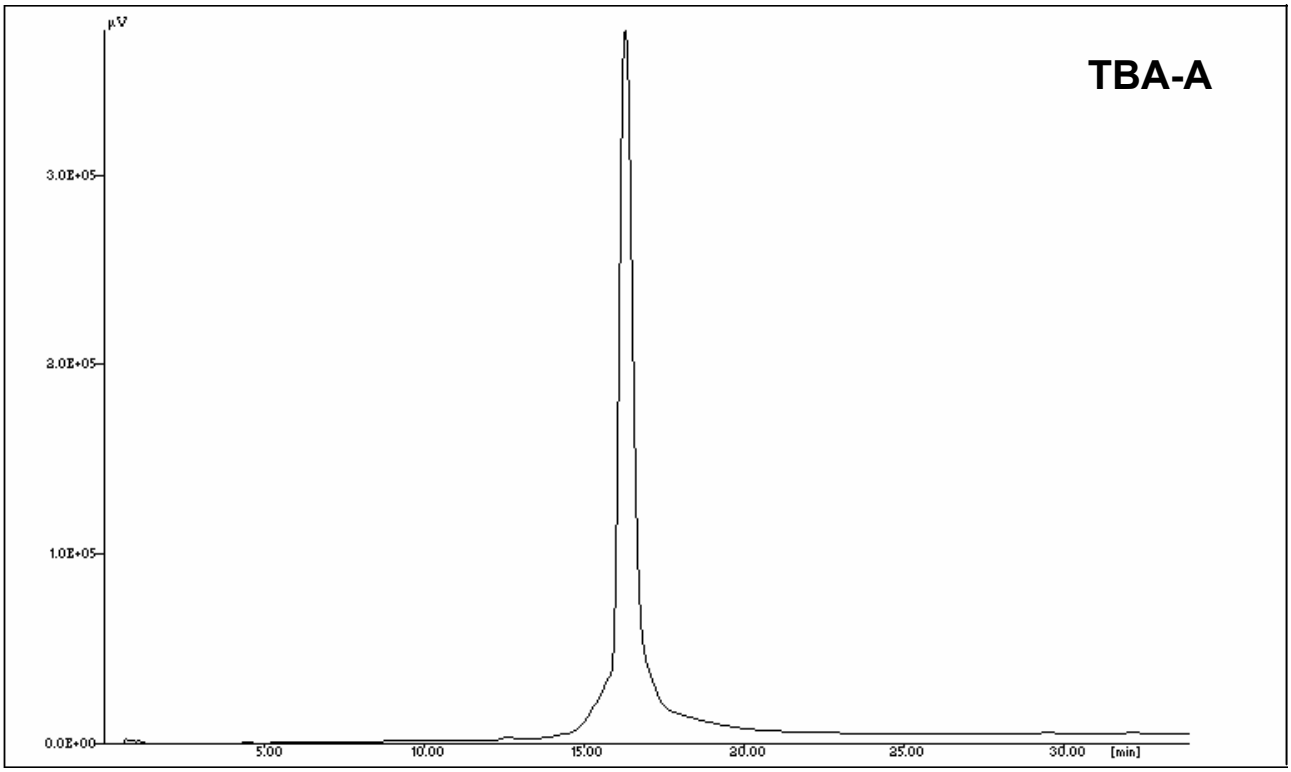


Figure S5. Aromatic region of the high resolution NMR spectra (700 MHz, D_2O, 50°C, no salt) of A-5'-5'-GGTTGGTGTGGTTGG-3'-3'-T (**A**), C-5'-5'-GGTTGGTGTGGTTGG-3'-3'-G (**B**), G-5'-5'-GGTTGGTGTGGTTGG-3'-3'-C (**C**) and T-5'-5'-GGTTGGTGTGGTTGG-3'-3'-A (**D**).



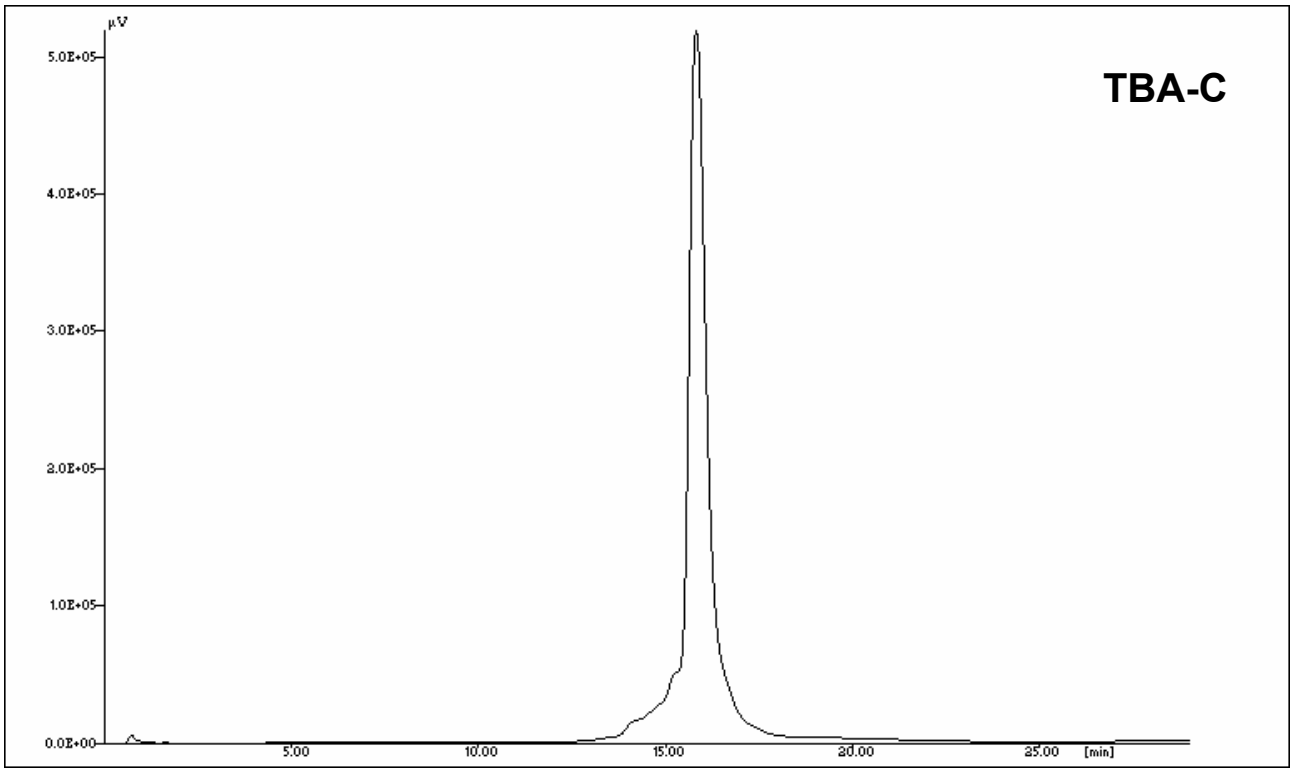
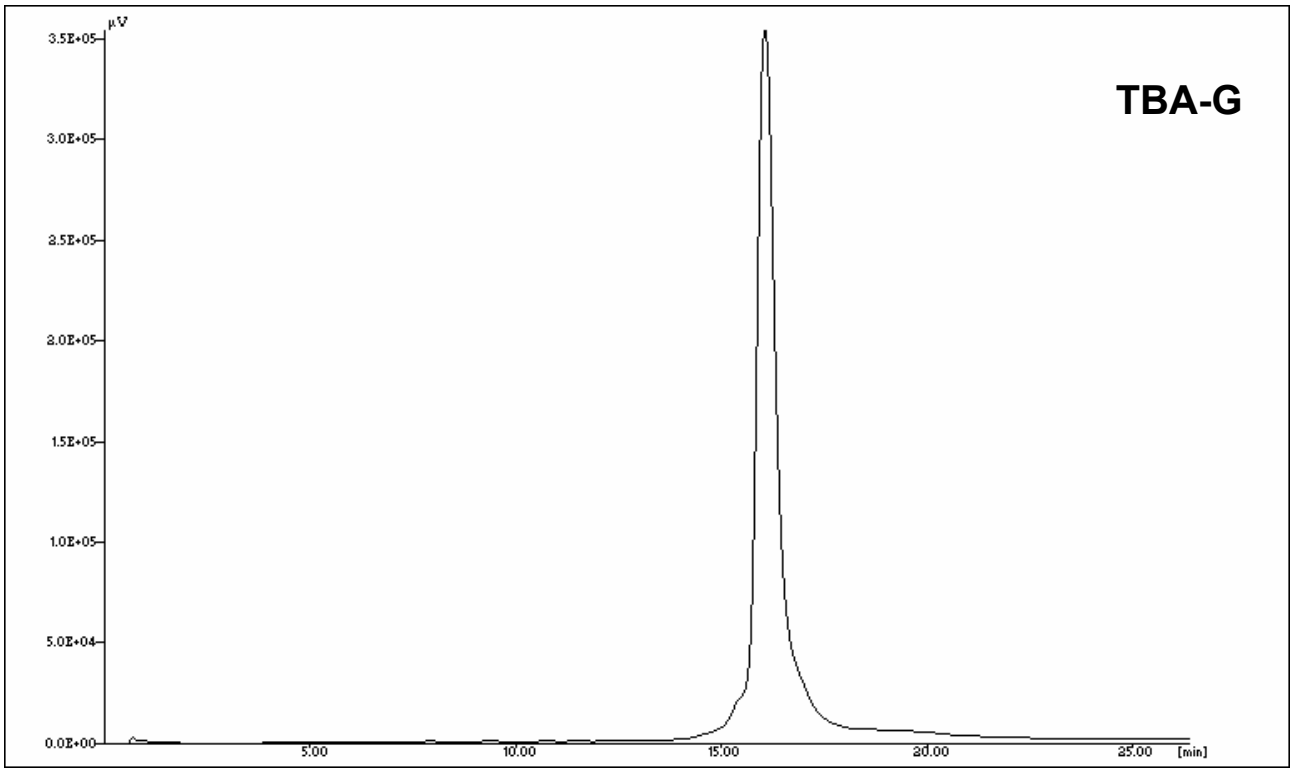


Figure S6. HPLC chromatograms of TBA-A and TBA-C. See experimental section for details.



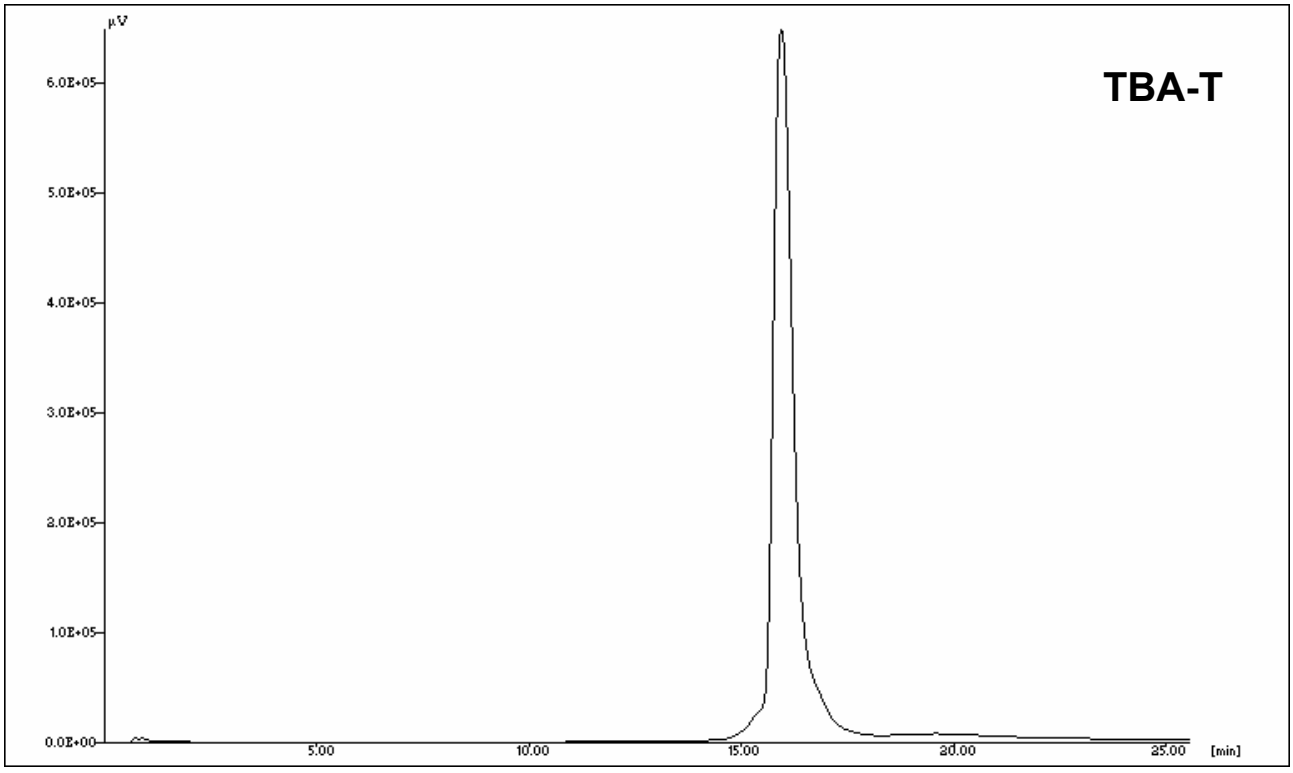
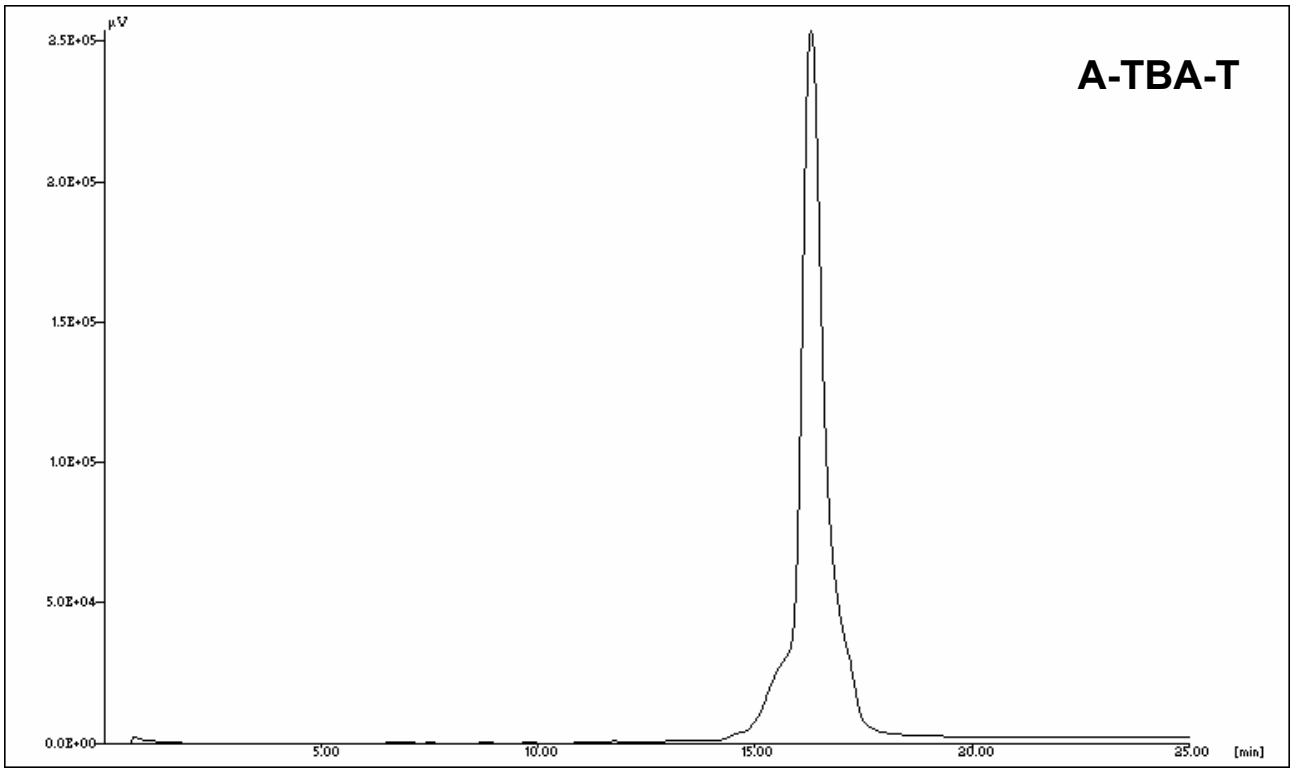


Figure S7. HPLC chromatograms of TBA-G and TBA-T. See experimental section for details.



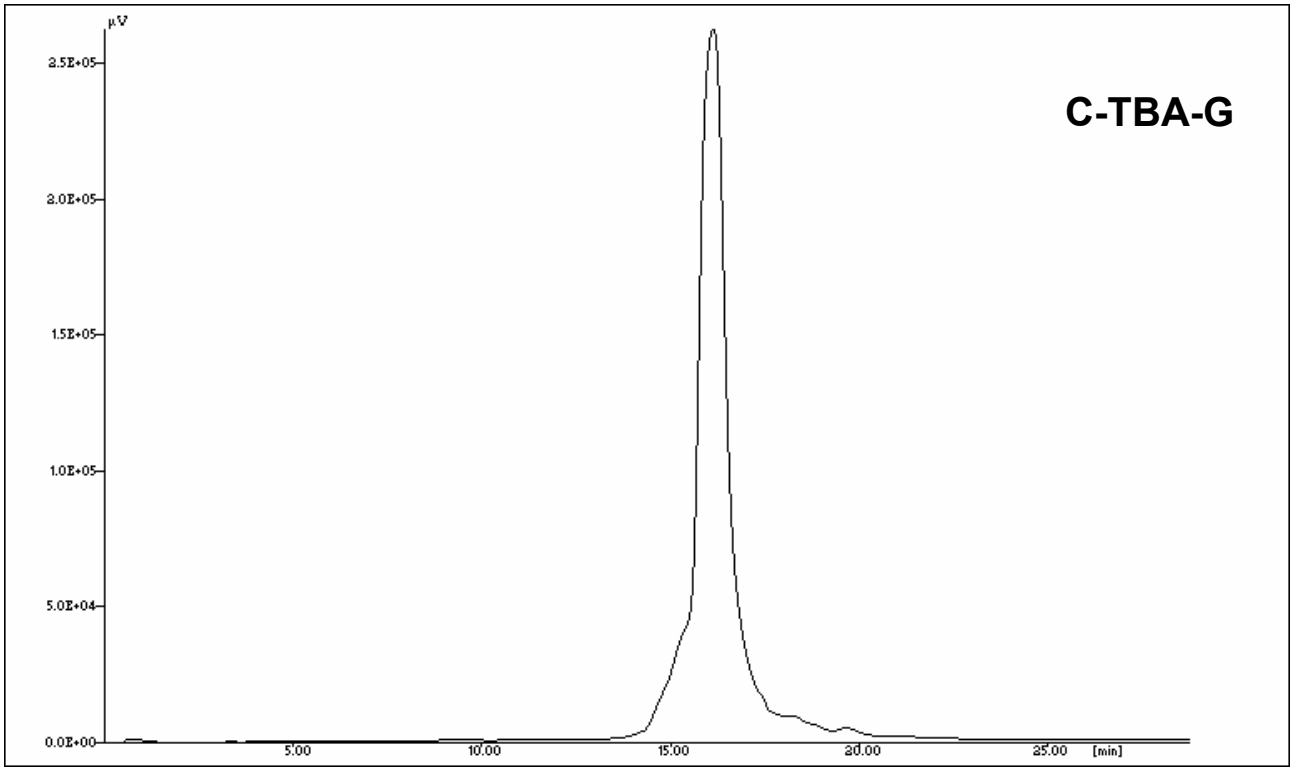
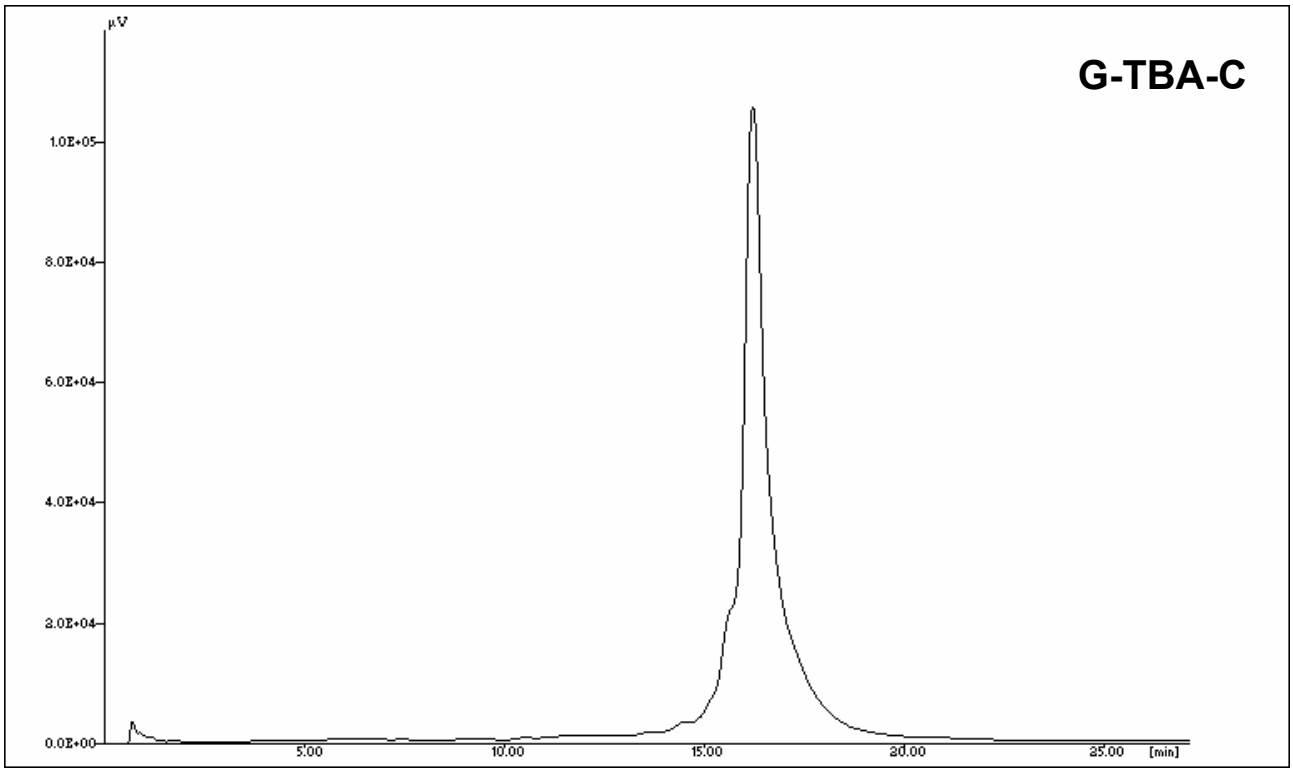


Figure S8. HPLC chromatograms of A-TBA-T and C-TBA-G. See experimental section for details.



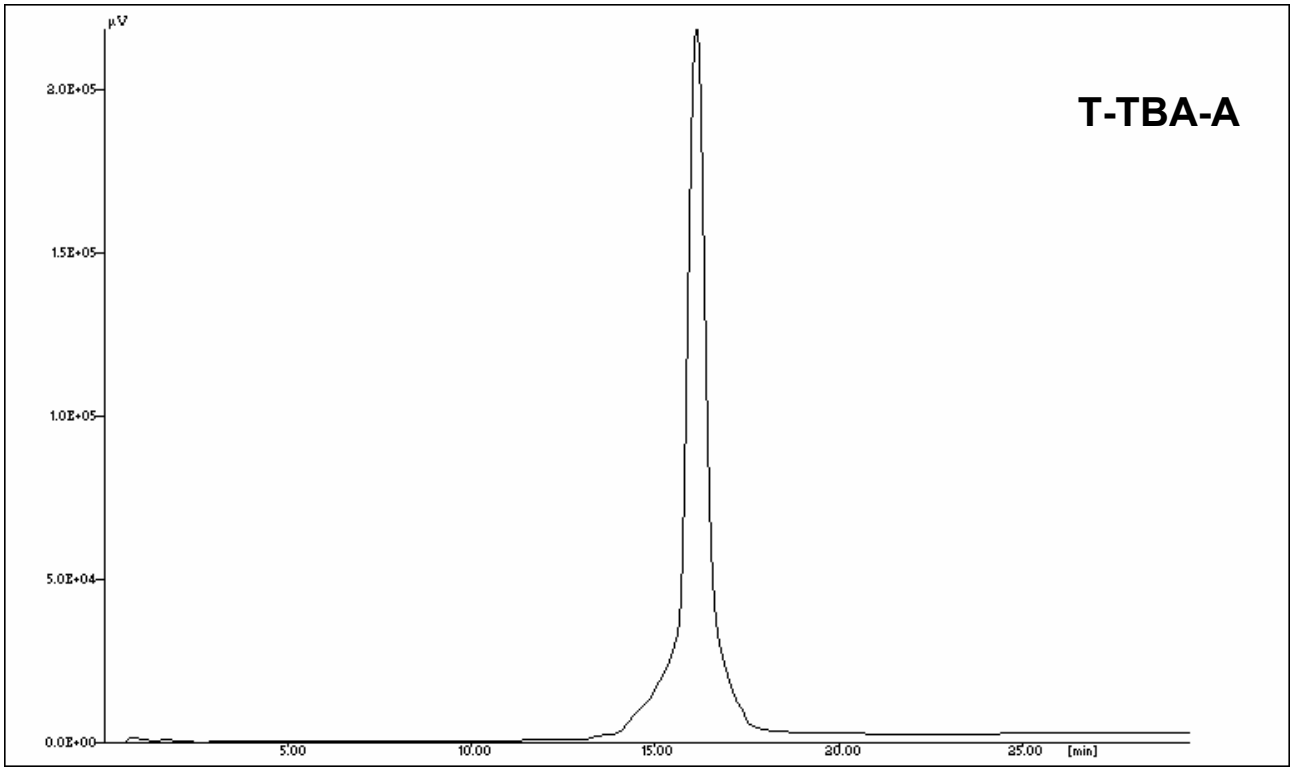


Figure S9. HPLC chromatograms of G-TBA-C and T-TBA-A. See experimental section for details.