Supporting information for

Phosphorothioate modification improves exon-skipping of antisense oligonucleotides based on sulfonyl phosphoramidates in *mdx* mouse myotubes

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1. Synthesis and purification of chemically modified AOs

4-(Azidosulfonyl)-*N,N,N*-trimethylbutan-1-aminium iodide[1] and tosyl azide (*p*-toluenesulfonyl azide, TsN_3)[2] were synthesised as described previously and used for the synthesis of chemically modified AOs.[1, 3] One should note that TsN_3 should be handled with caution as it has the shock sensitivity of tetryl (*N*-methyl-*N*-2,4,6-tetranitroaniline) and the explosiveness of TNT.[4]

Modified 2′-OMe AOs (Table 1) with N+ and Ts- modifications were synthesised using a Mermaid-4 automated DNA synthesiser (BioAutomation Corp., USA) using 5-ethylthio-1*H*-tetrazole (ETT) as an activator. 2′-OMe phosphoramidites (Innovassynth Technologies Ltd., India) were used with increased coupling time (6 min) as well as oxidation and deprotection times (80 s, repeat 3 times) for 5 μmol scale synthesis. Freshly distilled MeCN was cooled to RT before use for the 2′-OMe phosphoramidites. As 5′-ODMT-2′-OMe-N^4-Ac-cytidine-3′-CEPA has limited solubility in MeCN and precipitates at low temperatures, it was dissolved in a 1:1 mixture of dry MeCN/CH_2Cl_2. 3-[(Dimethylamino-methylidene)amino]-3*H*-1,2,4-dithiazole-3-thione (DDTT, Sulfurizing Reagent II form GlenResearch, USA) was used for sulfurisation of AOs. The sulfurization step (2 - 4 min) was conducted before capping as a replacement of standard oxidation step. Standard oxidation and DMT-deprotection times were 60 s (repeat 3 times). Increased coupling time (6 min) was applied for 5 μmol synthesis scale. For incorporation of N+ and Ts modifications, the automatic synthesis was paused before the capping step (after coupling step and wash), the column was taken out from the DNA synthesiser and connected to a micro-tube pump. For N+ modification, 0.7 M 4-(azidosulfonyl)-*N*,*N*,*N*-trimethylbutan-1-aminium iodide in DMF (saturated and degassed, 2 mL) was pumped through the column for 30 min at 37 °C. For Ts- modification, TsN_3 in MeCN (0.5 M, 2 mL) was pumped through the column for 30 min at 37 °C. The column was placed back in the synthesiser to continue the synthesis.

After synthesis, the resulting ONs were cleaved from the solid support, and deprotected with concentrated aqueous ammonia (~28 %) at 55 °C for 8 hrs.

Purification of AOs was accomplished by HPLC using IE-column (TSKgel Super Q-5PW) and depending on the modification implemented, different mobile phases were applied. For AOs without PS linkages, buffer A (20 mM Tris-HCl, 1 mM Na_2-EDTA, pH 9.0) and buffer B (20

mM Tris-HCl, 1 mM Na_2-EDTA, 1M NaCl, pH 9.0) were used. For AOs with PS linkages, buffer A (25 mM Tris-HCl, 10 mM $NaClO_4$, 20% acetonitrile, pH 7.4) and buffer B (25 mM Tris-HCl, 600 mM $NaClO_4$, 20% acetonitrile, pH 7.4) were used as a mobile phase. Gradients were adjusted based on the type and number of modifications incorporated (Table S1). Collected individual UV-absorbing fractions (λ = 260 nm) were desalted by RP-HPLC on 250/4.6 mm, 5 µm, 300 Å C18 column (Phenomenex) in a gradient of CH_3CN (0→75% for 2 min, then 3 min at 75% of CH_3CN, 1.3 mL/min) in water. Isolated fractions were quantified and analysed by ESI-MS. All AOs are detected as individual species in ESI-MS without any n±1 or PS(±1) impurities. IE-HPLC profiles of crude mixtures and RP-HPLC profiles of desalted fractions are provided in Figures S1-S18 below.

Table S1. Retention time on IE-column and observed yield of AOs used in this study.

Abbreviation	Mobile phase	Gradient	Retention time (min)	Isolated yield (%)
2′-OMe RNA control	A: 20 mM Tris-HCl (1 mM Na_2-EDTA, pH 9.0) B: 20 mM Tris-HCl (1 mM Na_2-EDTA, 1M NaCl, pH 9.0)	0.3 — 0.5 M NaCl in 18.5 min	25.78	-
5N+AO1			18.68	1.4
4N+AO2			20.66	0.8
5Ts-AO3			40.13	5.1
4Ts-AO4			38.78	3.2
7N+AO5			15.40	0.5
7Ts-AO6		0.4 — 0.75 M NaCl in 23.9 min	37.82	0.3
5N+5PS-AO7	A: 25 mM Tris-HCl (10 mM $NaClO_4$, 20% Acetonitrile, pH 7.4) B: 25 mM Tris-HCl (600 mM $NaClO_4$, 20% Acetonitrile, pH 7.4)	0 — 0.24 M $NaClO_4$ in 55.5 min	41.57	0.9
5Ts-5PS-AO8		0.18 — 0.3 M $NaClO_4$ in 18.5 min	19.32	2.5
5N+all PS-AO9		0 — 0.24 M $NaClO_4$ in 55.5 min	57.10	0.9
5Ts-all PS-AO10		0.18 — 0.3 M $NaClO_4$ in 18.5 min	31.84	2.7

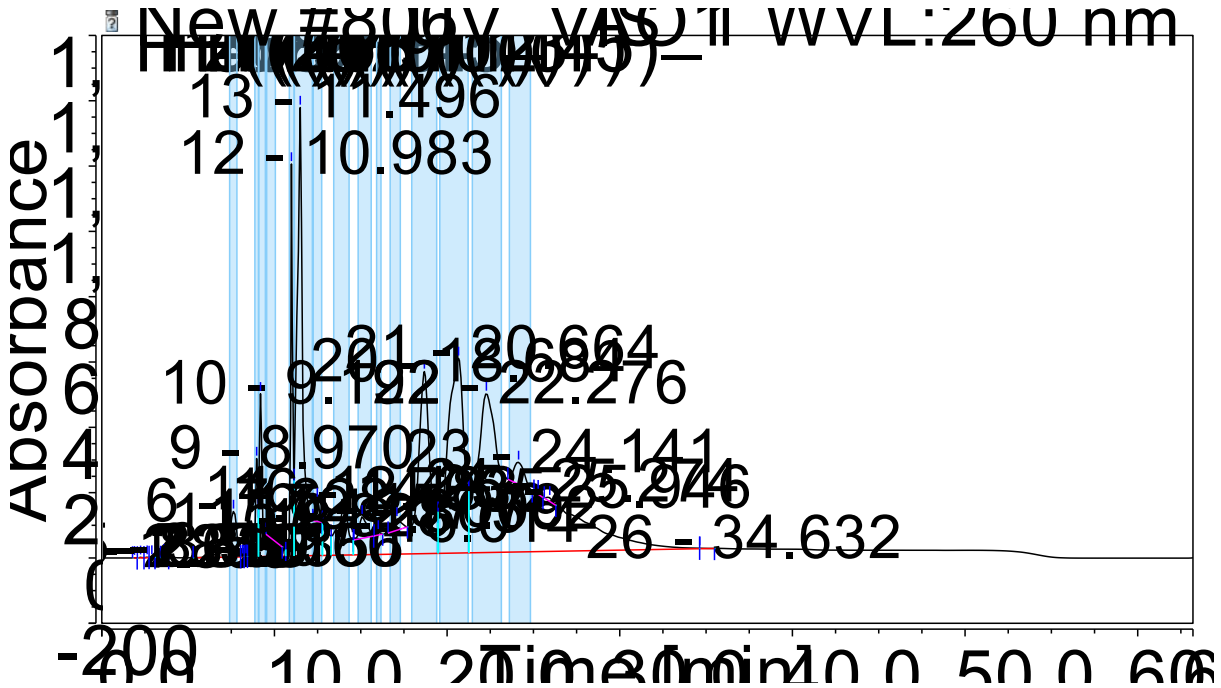

Figure S1. Crude IE-HPLC profile of 5N+AO1 (peak collected at 18.68 min) and 4N+AO2 (peak collected at 20.66 min).

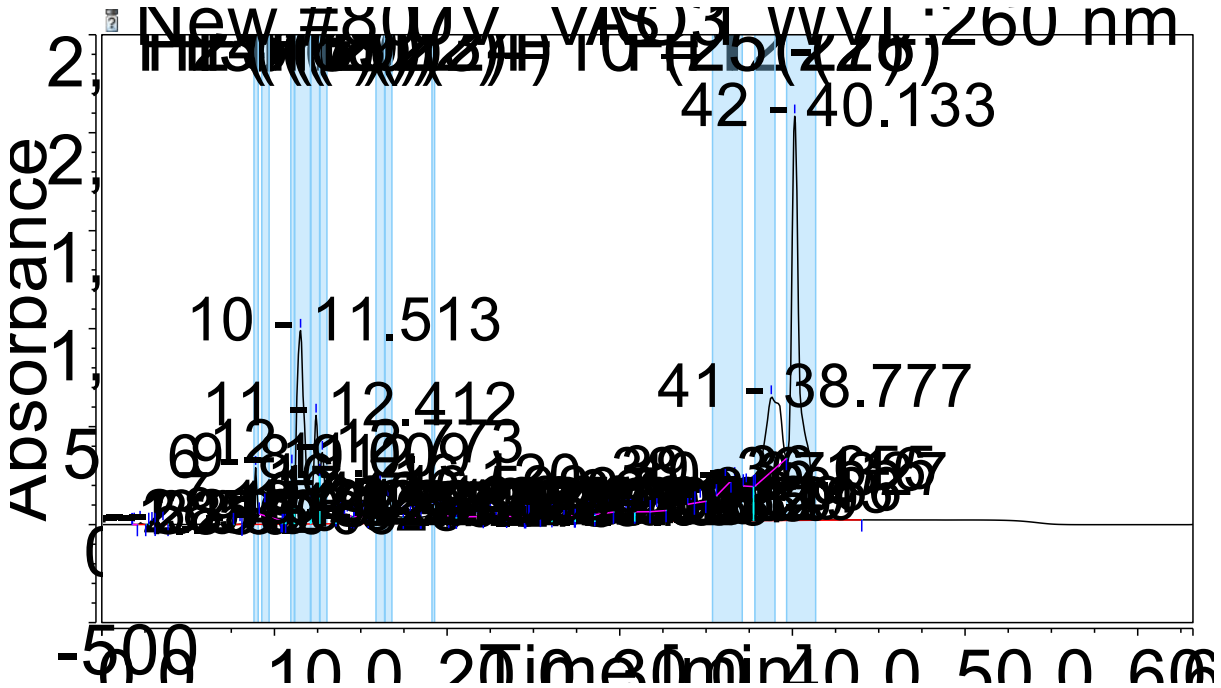

Figure S2. Crude IE-HPLC profile of 5Ts-AO3 (peak collected at 40.13 min) and 4Ts-AO4 (peak collected at 38.78 min).

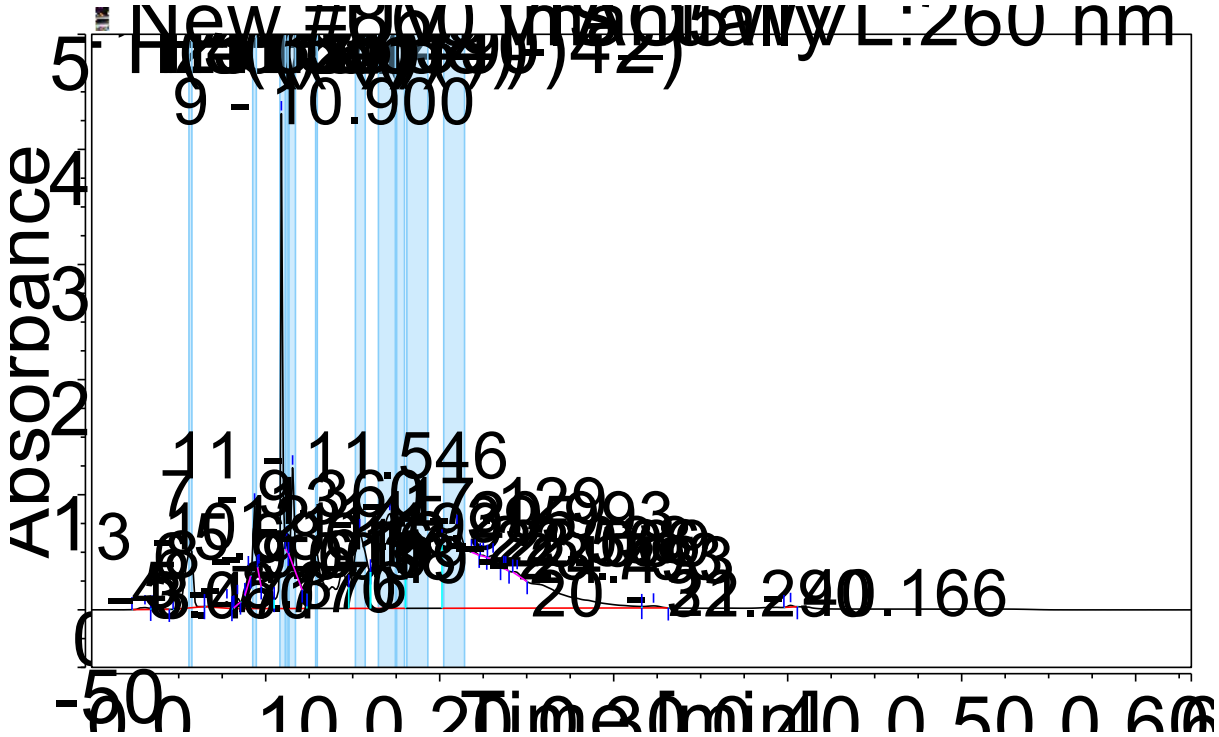

Figure S3. Crude IE-HPLC profile of 7N+AO5 (peak collected at 15.395 min).

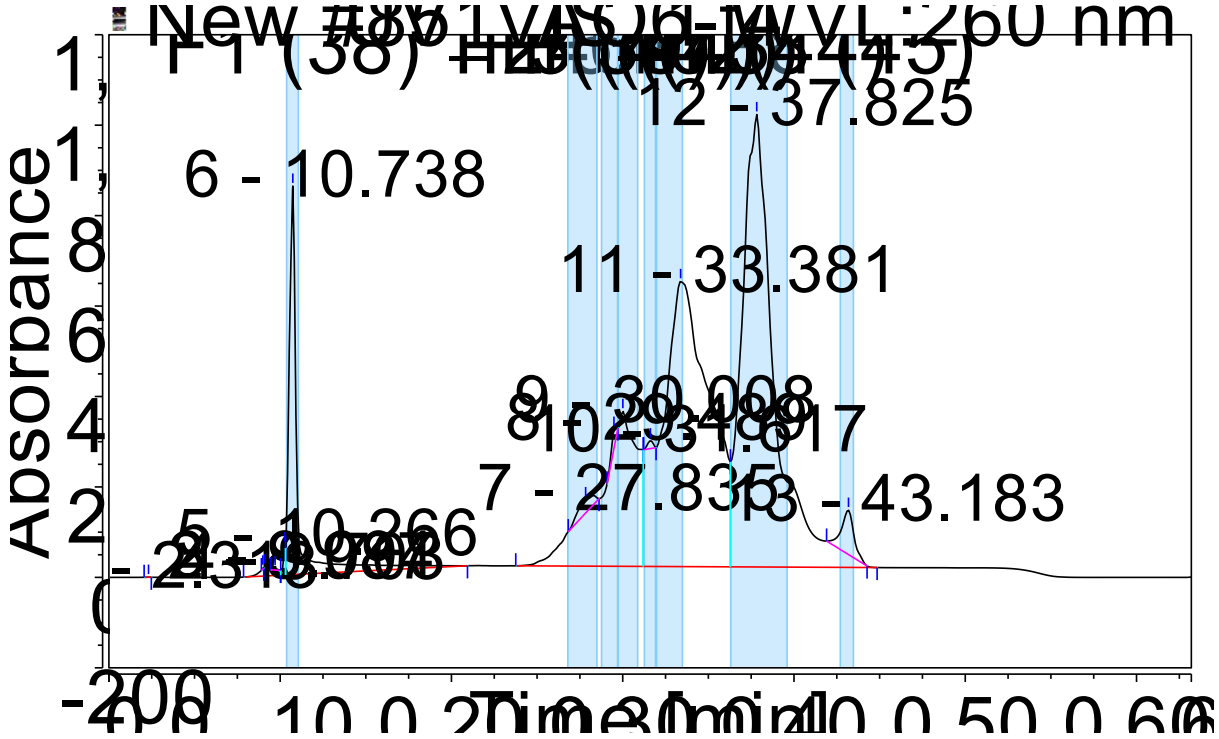

Figure S4. Crude IE-HPLC profile of 7Ts-AO6 (peak collected at 37.82 min).

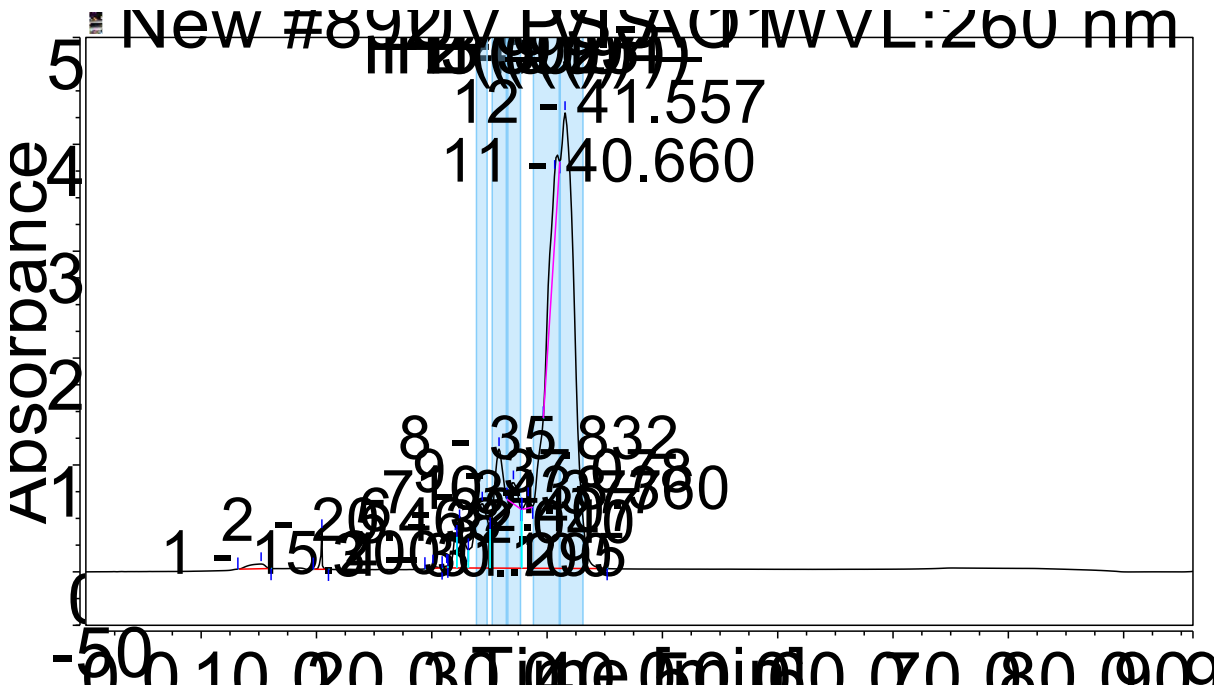

Figure S5. Crude IE-HPLC profile of 5N+5PS-AO7 (peak collected at 41.56 min).

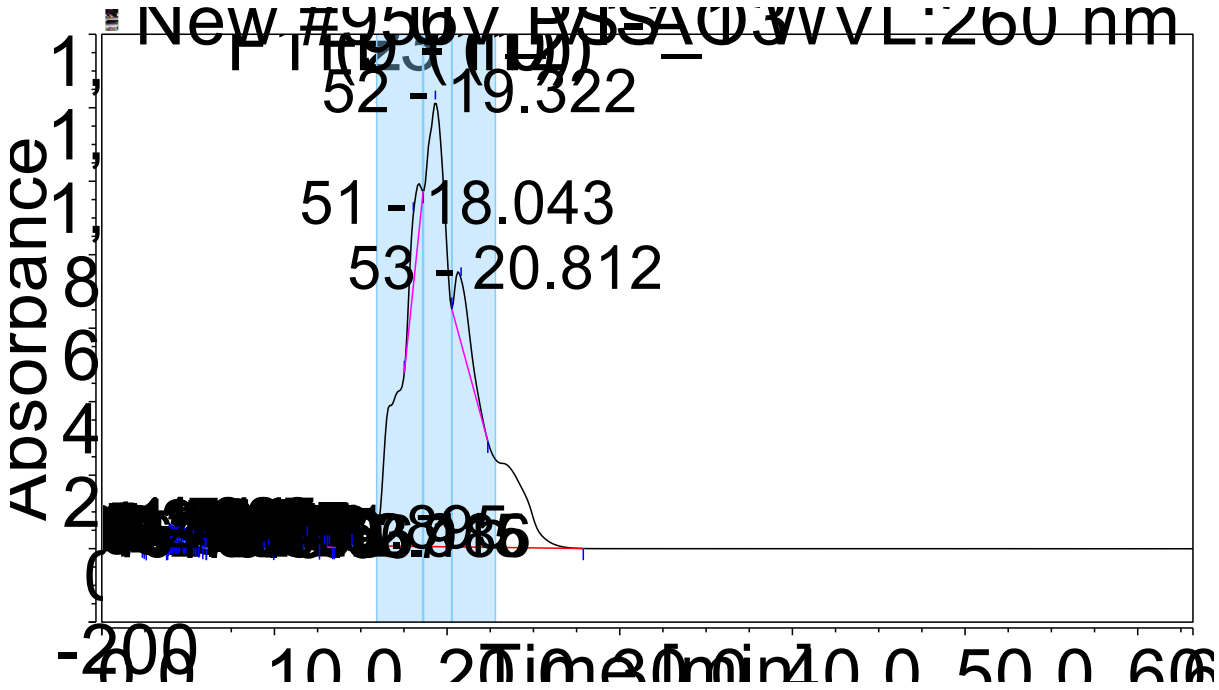

Figure S6. Crude IE-HPLC profile of 5Ts-5PS-AO8 (peak collected at 19.32 min).

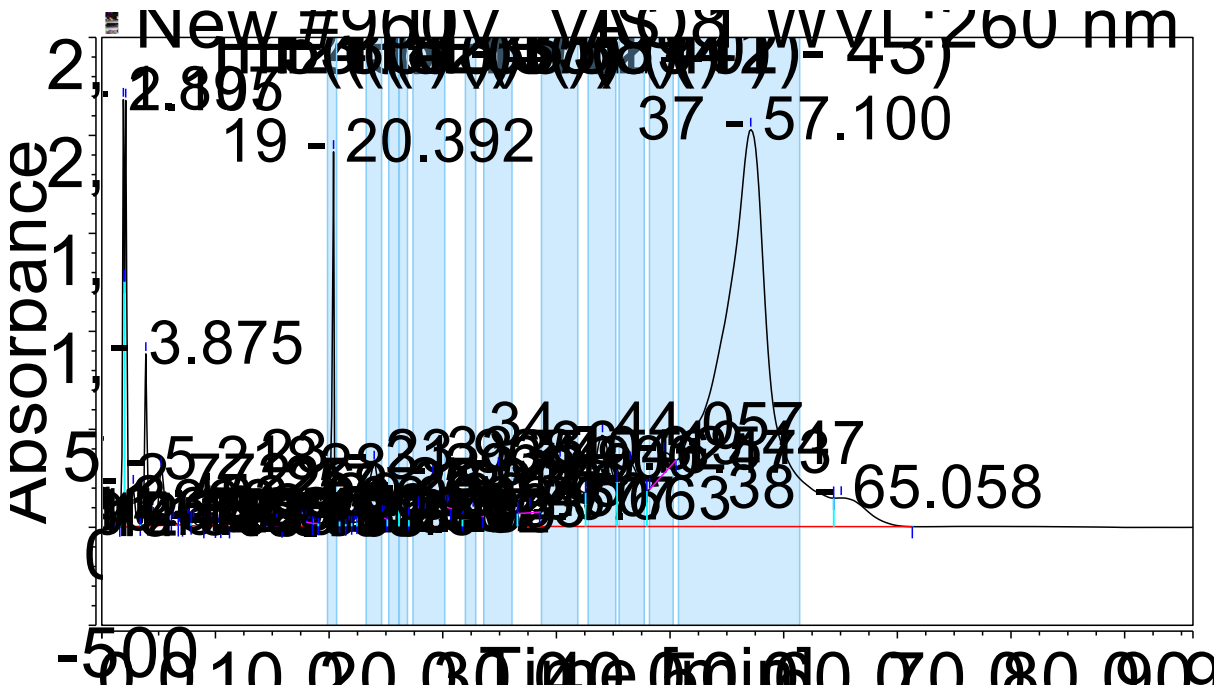

Figure S7. Crude IE-HPLC profile of 5N+allPS-AO9 (peak collected at 57.10 min).

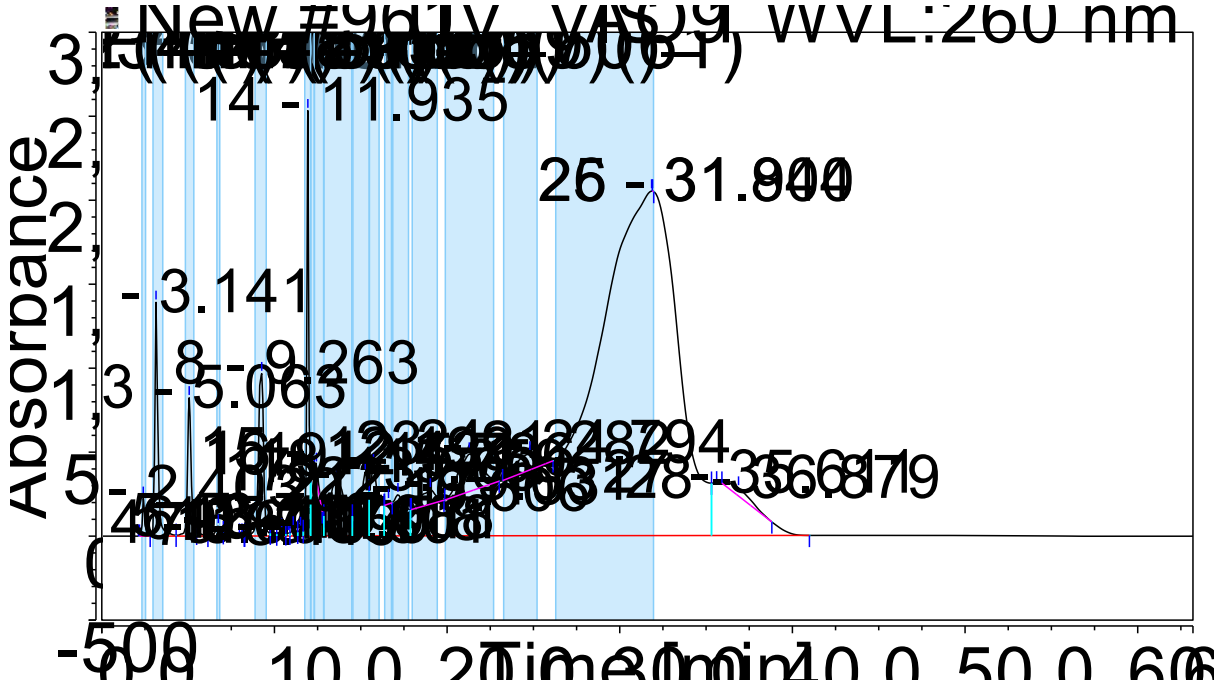

Figure S8. Crude IE-HPLC profile of 5Ts-allPS-AO10 (peak collected at 31.84 min).

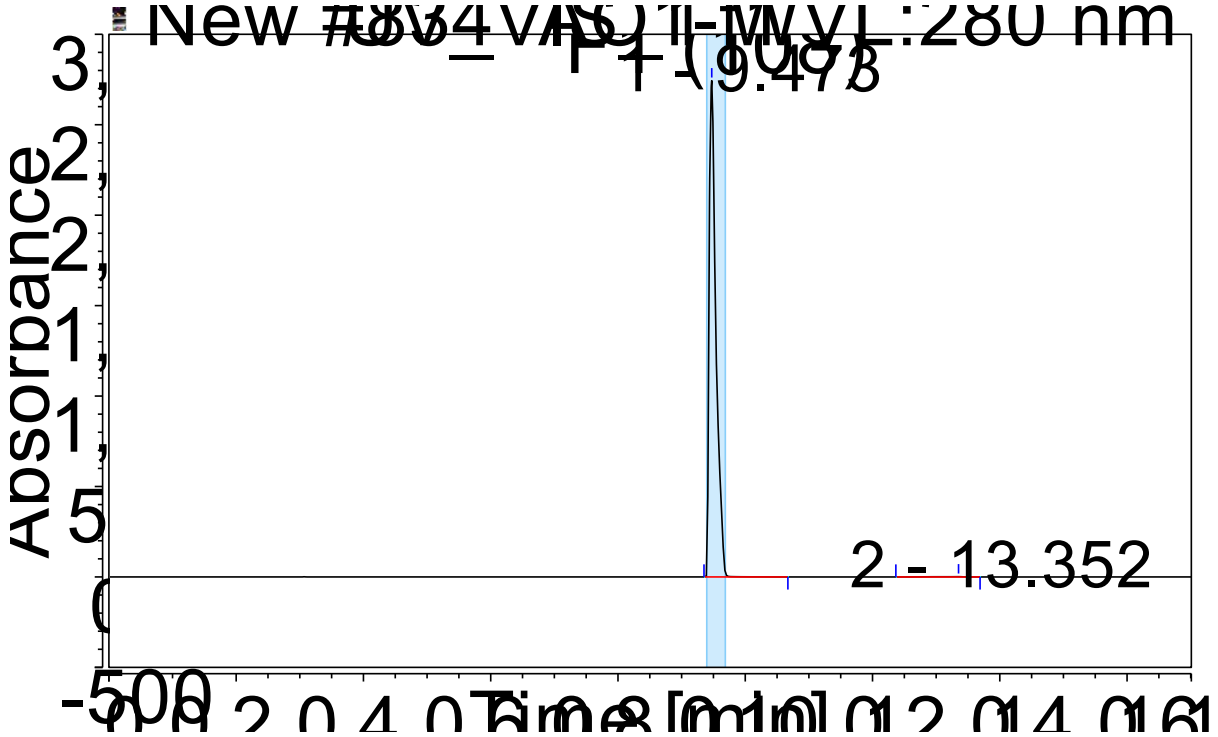

Figure S9. RP-HPLC of 5N+AO1.

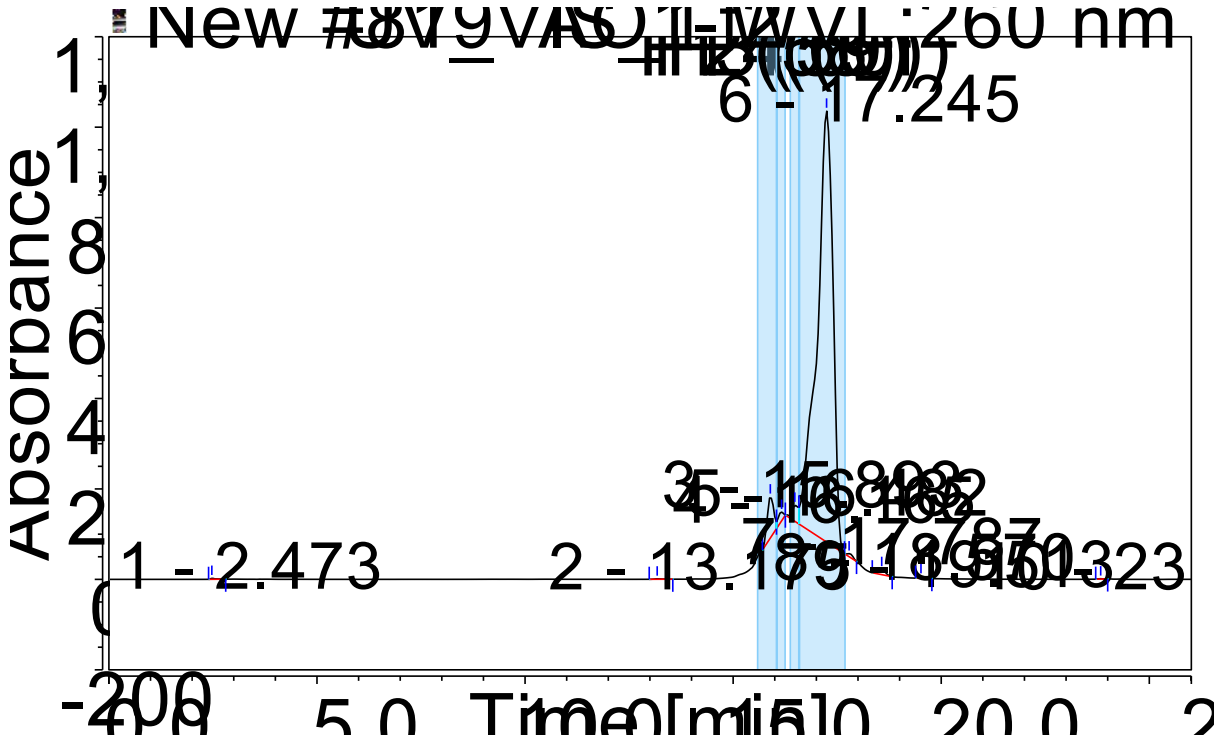

Figure S10. RP-HPLC of 4N+AO2.

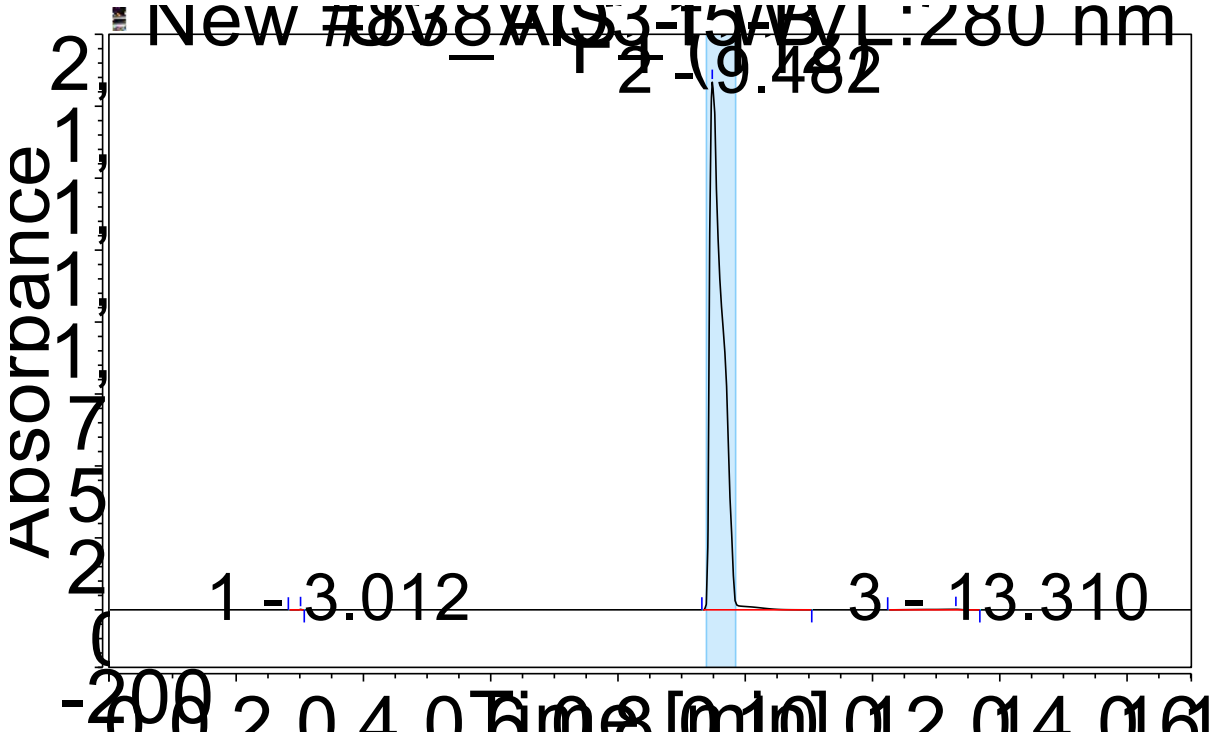

Figure S11. RP-HPLC of 5Ts-AO3.

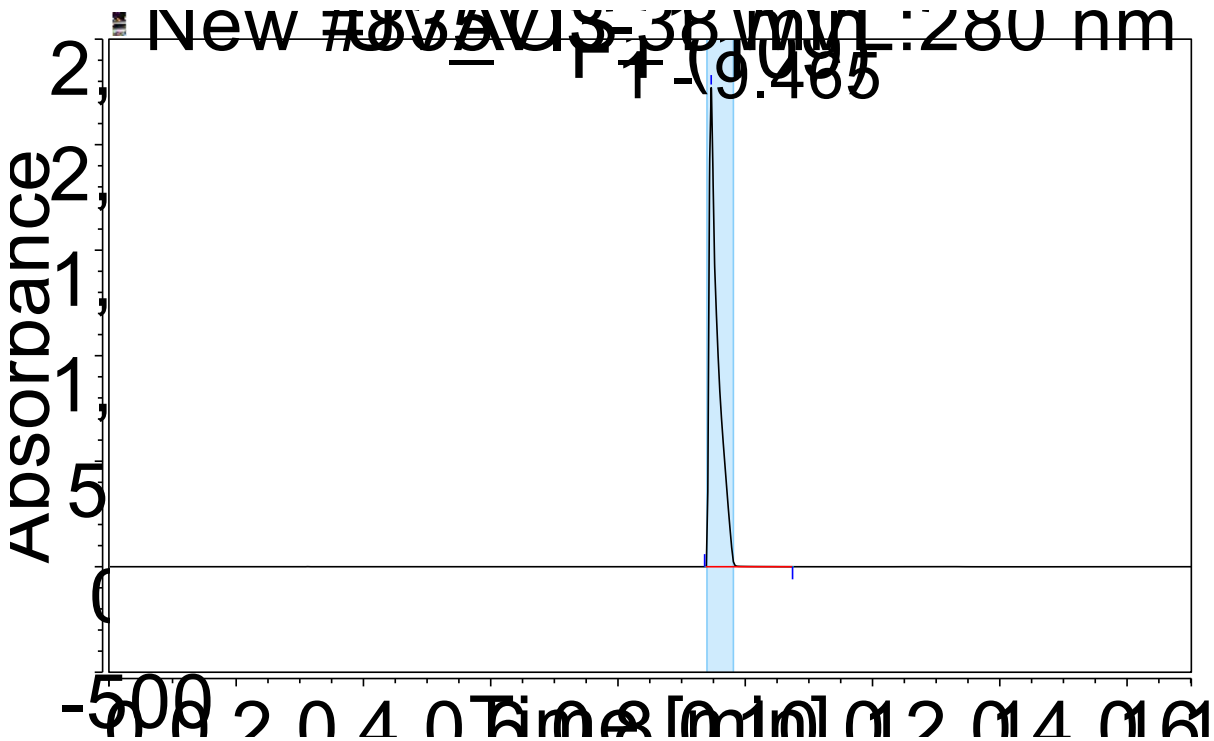

Figure S12. RP-HPLC of 4Ts-AO4.

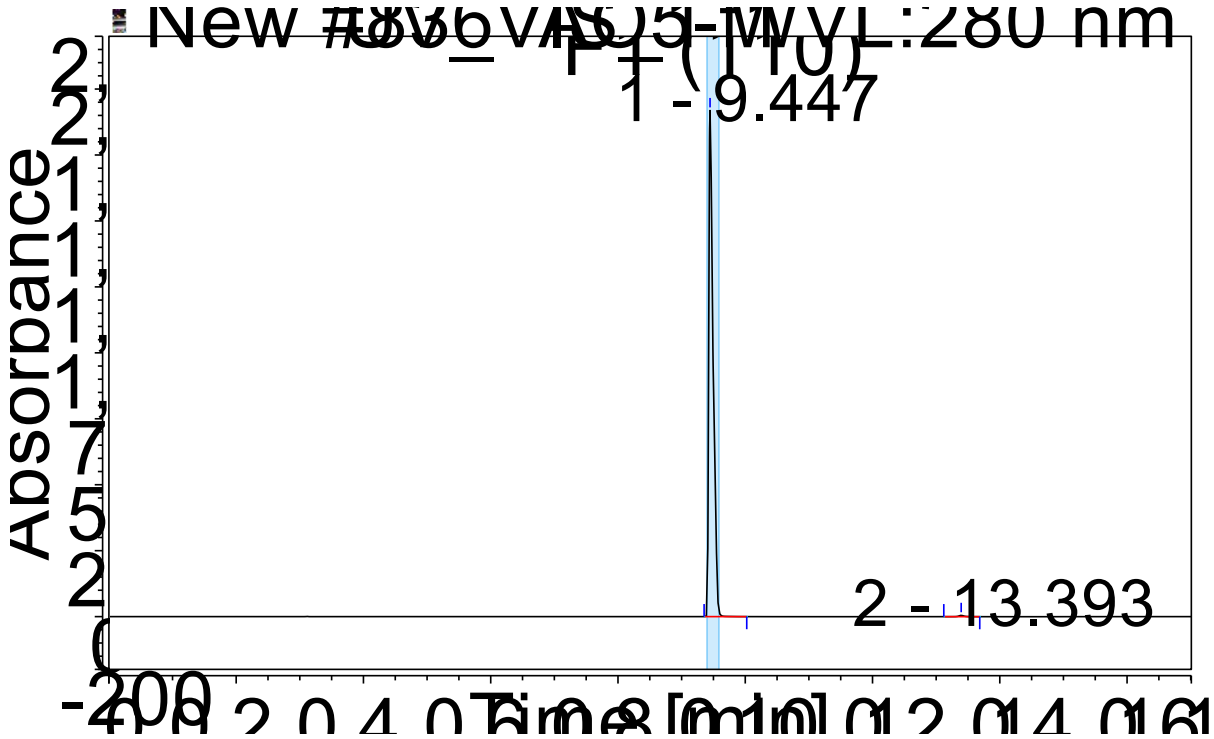

Figure S13. RP-HPLC of 7N+AO5.

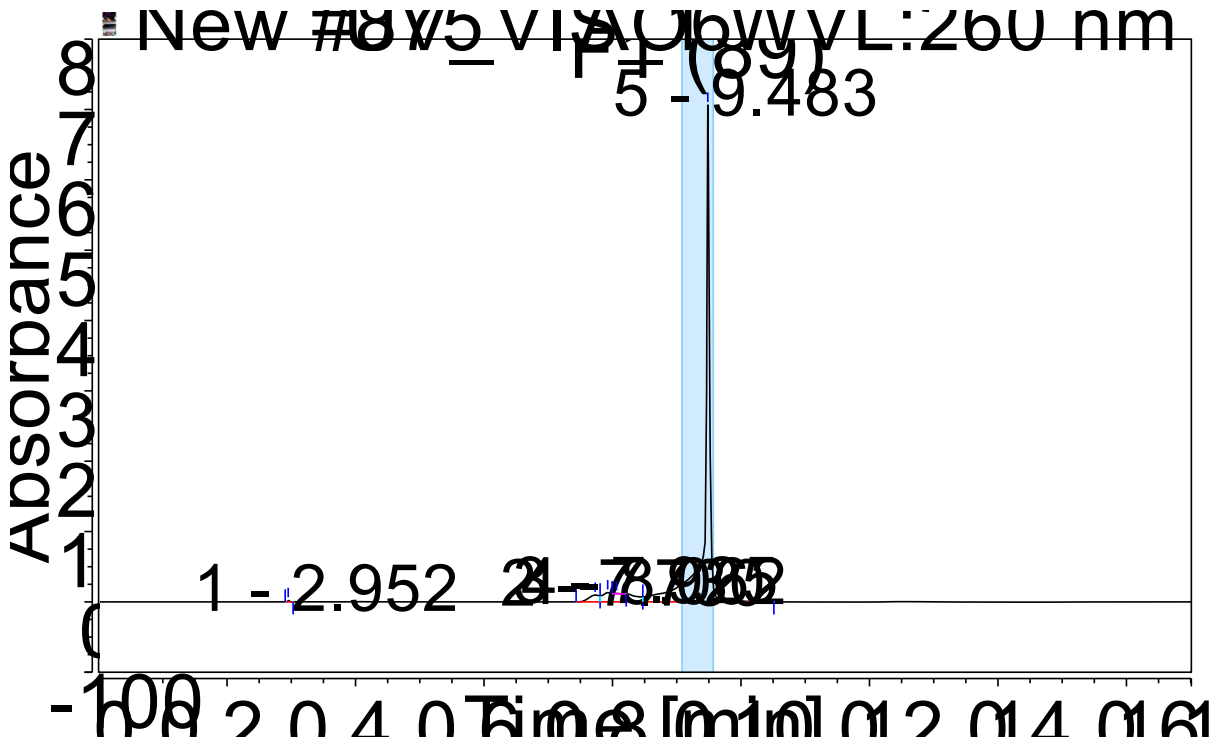

Figure S14. RP-HPLC of 7Ts-AO6.

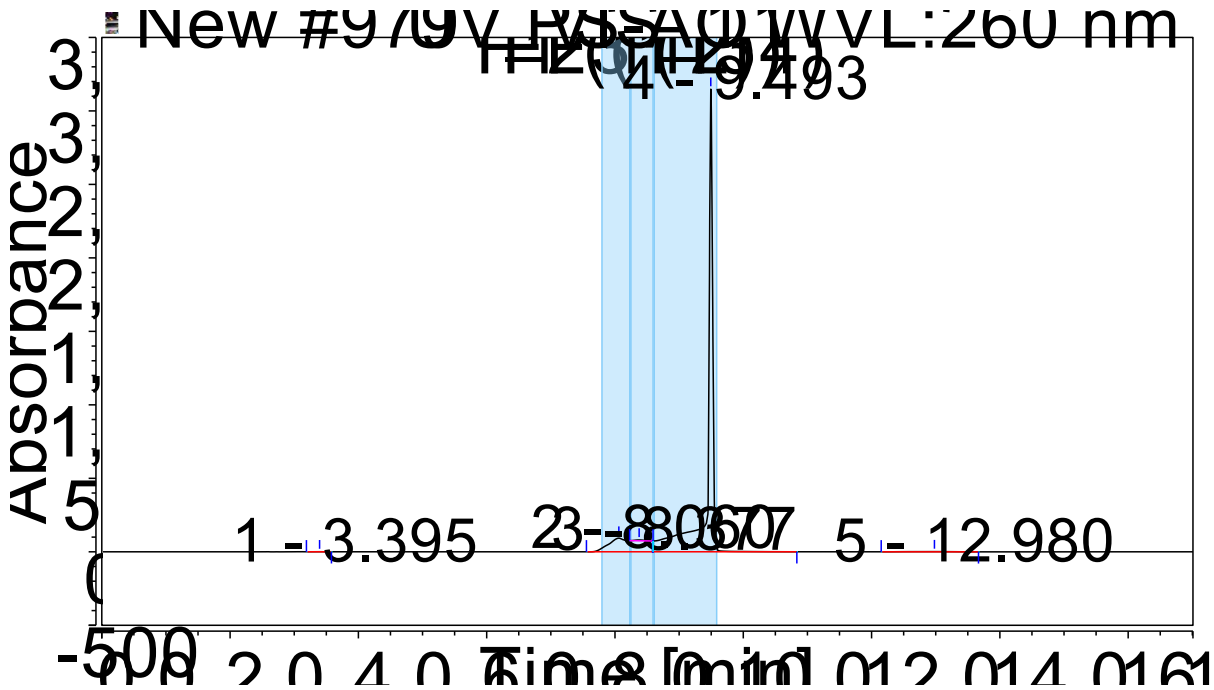

Figure S15. RP-HPLC of 5N+5PS-AO7.

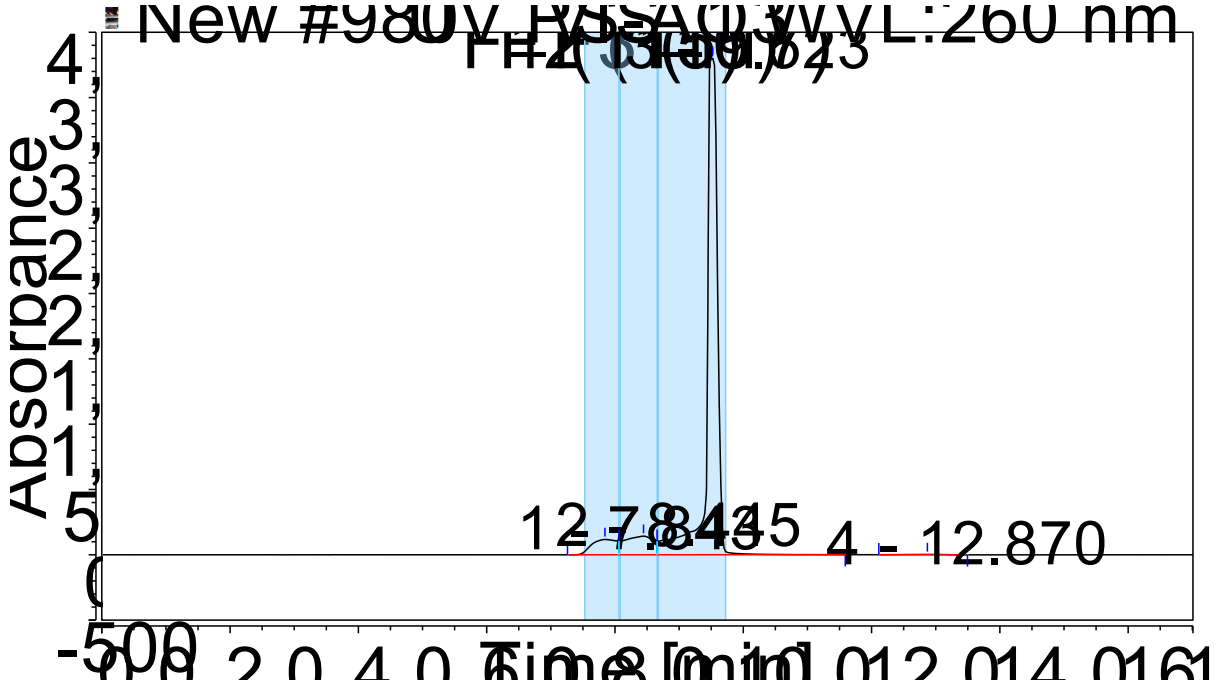

Figure S16. RP-HPLC of 5Ts-5PS-AO8.

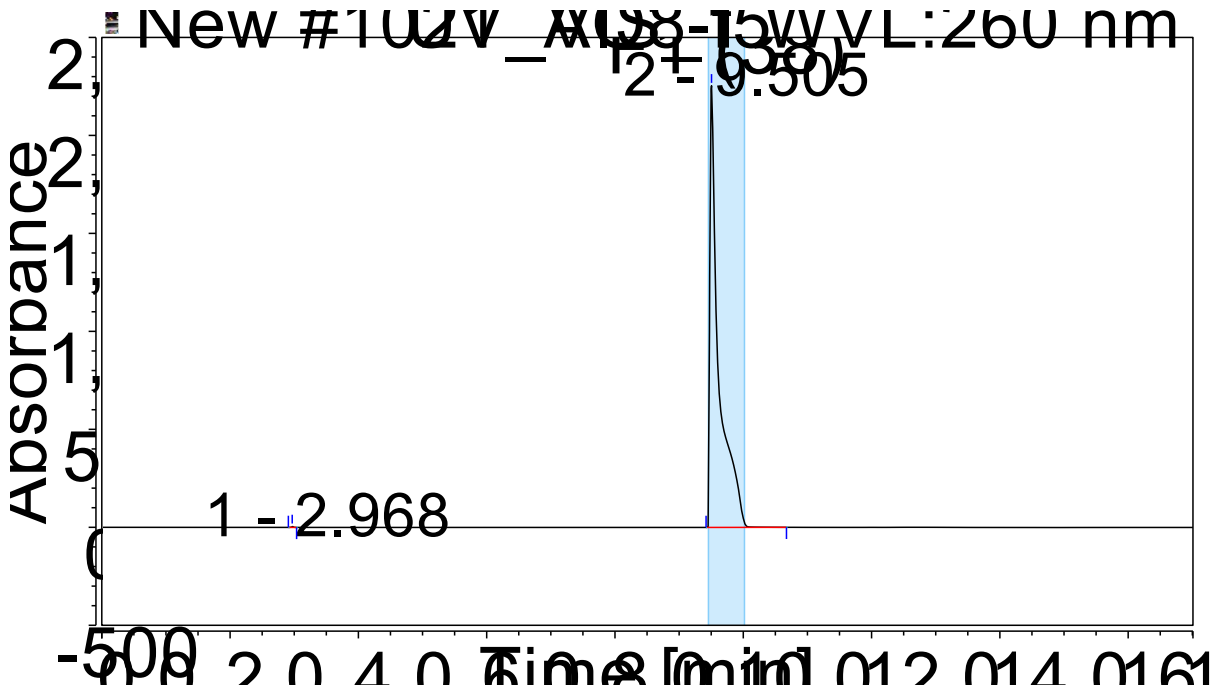

Figure S17. RP-HPLC of 5N+allPS-AO9.

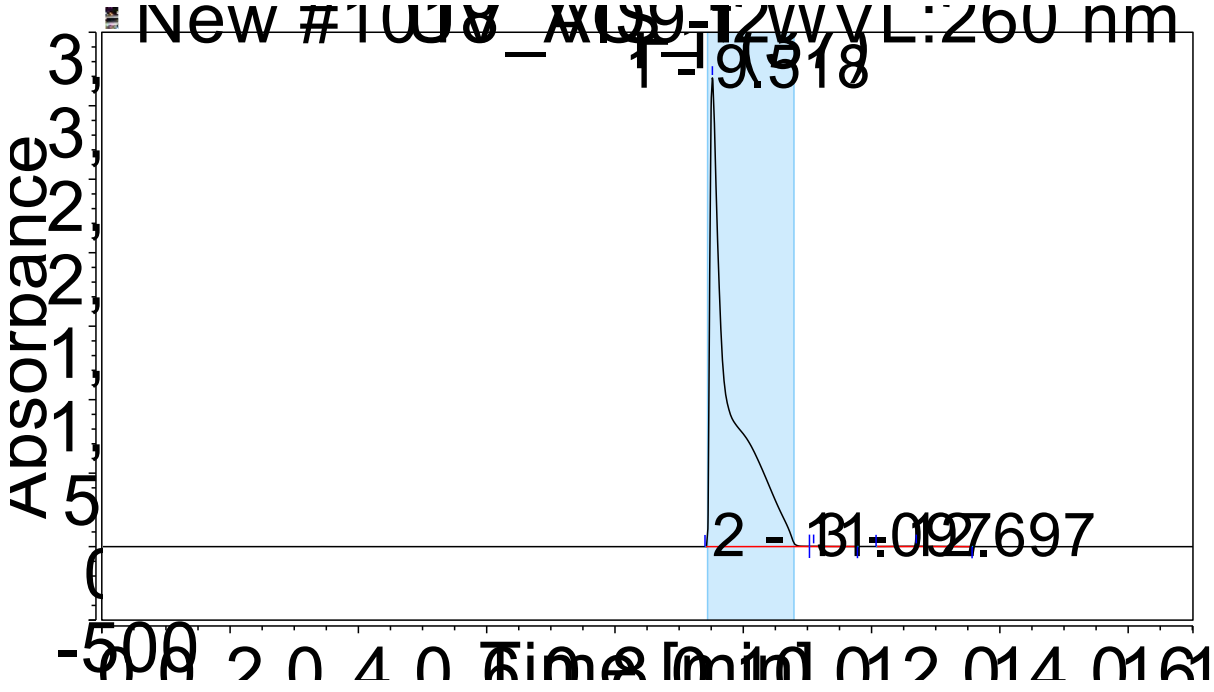

Figure S18. RP-HPLC of 5Ts-allPS-AO10.

2. ESI-MS analysis of modified AOs

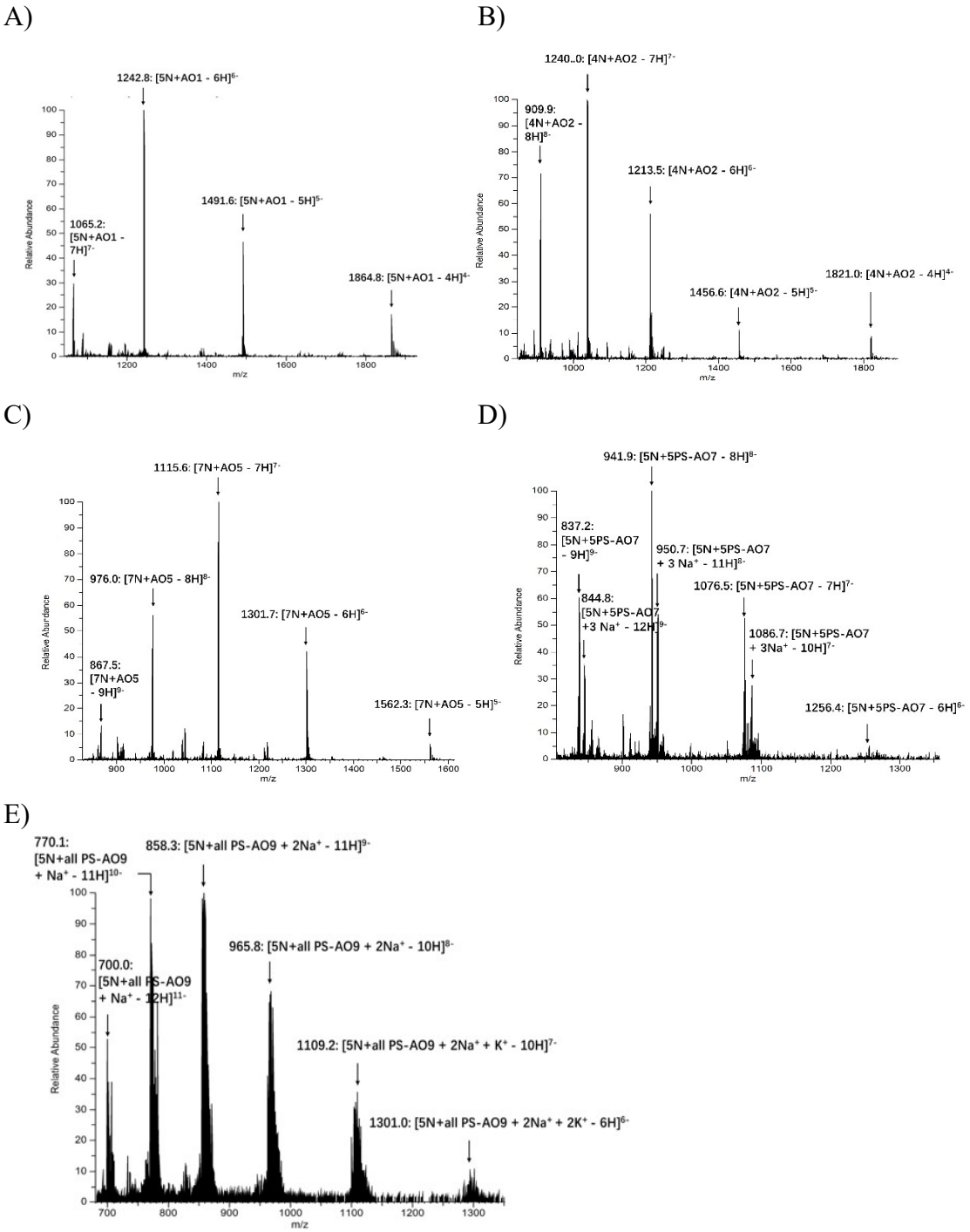

Figure S19. ESI-MS spectra of N+ modified AOs summarised in Table 1 in the main text.

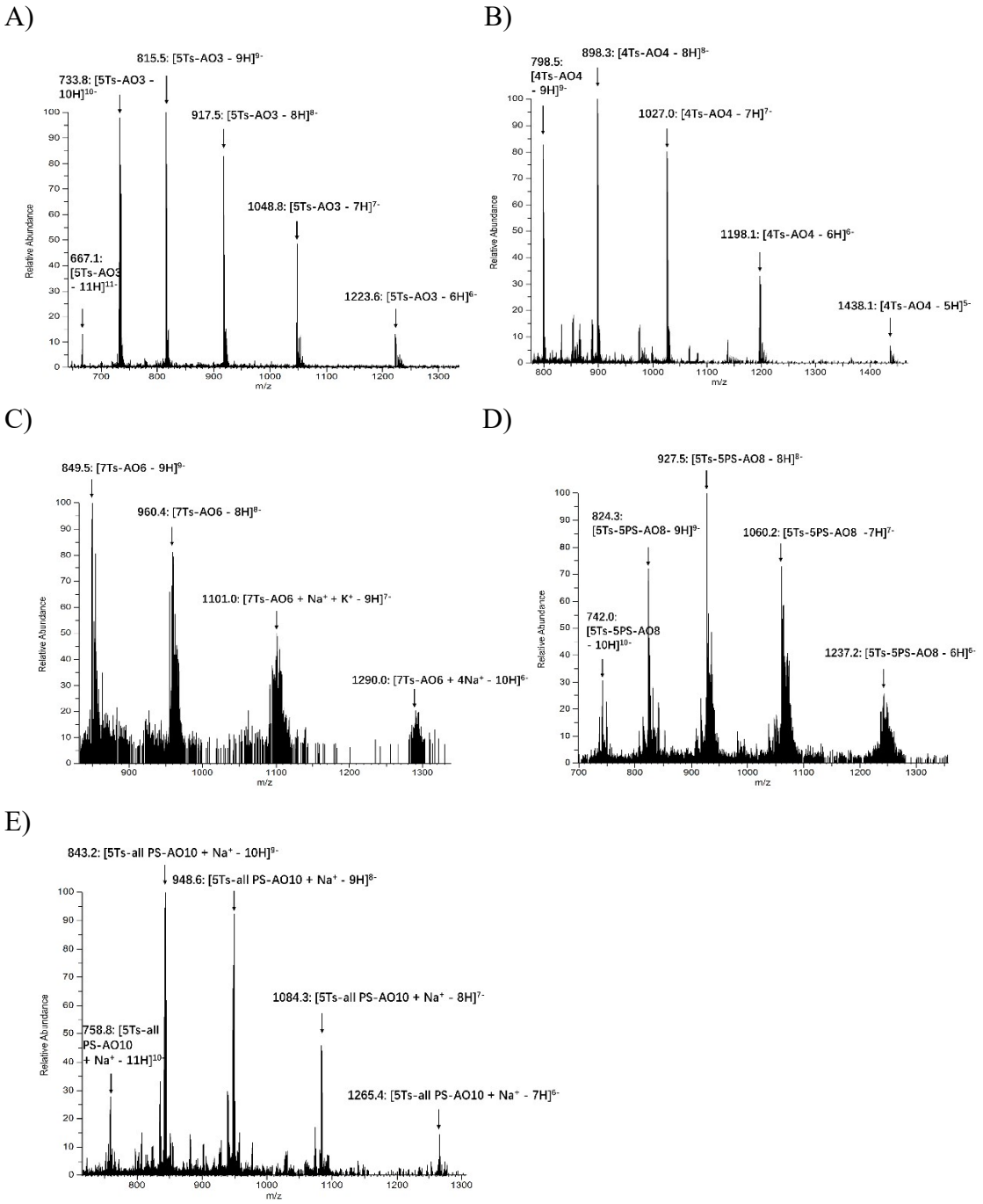

Figure S20. ESI-MS spectra of Ts-modified AOs summarised in Table 1 in the main text.

3. Thermal stability assessment of chemically modified AOs against RNA target

UV-Vis spectroscopy was performed using a Cary 100Bio UV-Vis spectrometer and quartz cuvettes with 1 cm path length and a 2 x 6 multicell block with a Peltier temperature controller. DNA melting profiles were recorded by monitoring absorbance at 260 nm using a 1 °C min^{-1} temperature ramp. Duplexes were prepared at 2.0 μM strand concentrations in 5 mM sodium phosphate buffer (70 mM NaCl, 0.05 mM Na_2-EDTA, pH 7.0). The melting temperatures (T_m [°C]) were determined as the maxima of the first derivative plots of the melting curves.[5]

Because duplexes of AOs and RNA target were not fully denatured at the salt concentration of 70 mM (Fig. S3A), we decided to halve the salt concentration in denaturation experiments. Under improved conditions (35 mM NaCl, 25 μM Na_2-EDTA, pH 7.0), all duplexes melted, and T_m values could be compared (Fig. S3B).

We analysed melting profiles and obtained thermodynamic parameters of duplexes ignoring the changes in DNA and salt concentrations induced by solution evaporation, the change of pH during heating, and assuming that there is no change in the heat capacity ($\Delta C_p = 0$). We also assumed a two-state transition between duplex and single-stranded DNA or RNA and linear relationship between UV signal and fraction of molecules unfolded.[5] Thermodynamic parameters were analysed only for duplexes in which the start of the upper baseline was detectable. Melting curves were converted to fraction folded (α) versus T curves as specified in reference [5]. For extraction of thermodynamic parameters only α values significantly higher than 0 (higher baseline) and lower than 1 (lower baseline) can be used, which reflects the transition. These values were available for all duplexes except for three duplexes with T_m higher than 80 °C.

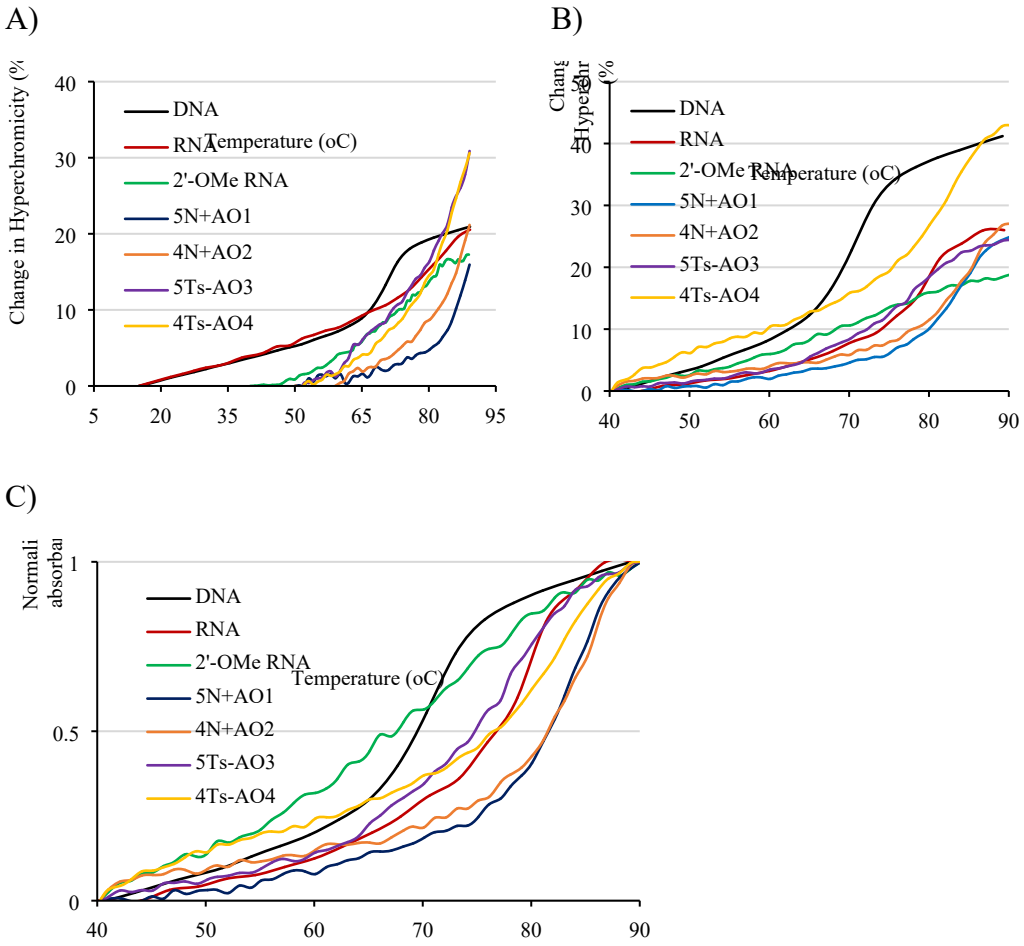

Figure S21. Melting profiles of duplexes formed by AOs having four and five N+ or Ts modifications in a complex with the RNA target. A) Change in hyperchromicity at 260 nm. Conditions: 2.0 µM of each strand in 5 mM sodium phosphate buffer (70 mM NaCl, 50 µM Na_2-EDTA, pH 7.0). B) Change in hyperchromicity at 260 nm. Conditions: 2.0 µM of each strand in 5 mM sodium phosphate buffer (35 mM NaCl, 50 µM Na_2-EDTA, pH 7.0). C) Normalised melting profiles of duplexes with RNA target present in B.

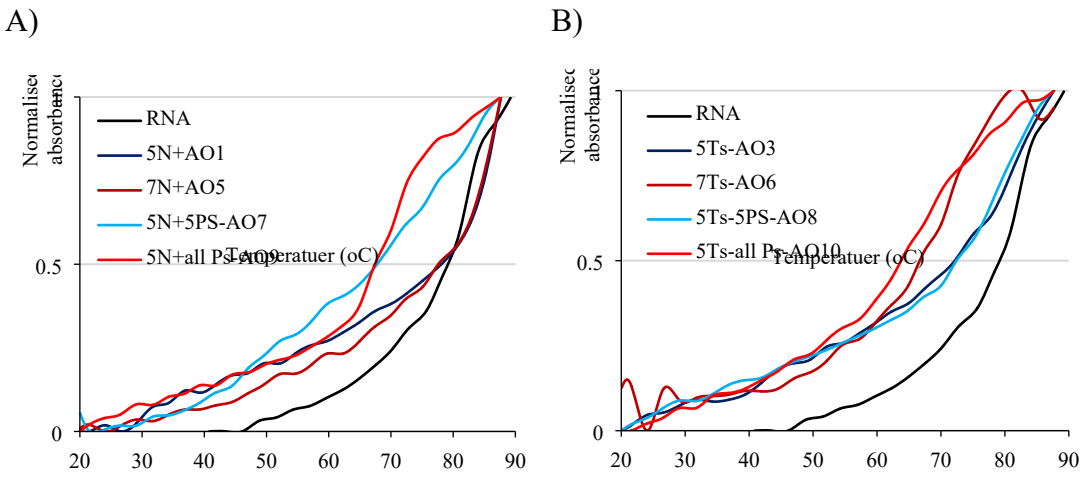

Figure S22. Melting profiles of duplexes having five and seven N+ or Ts modifications in AO in a complex with the RNA target. A) Normalised melting profiles of duplexes formed between N+AOs and RNA target. B) Normalised melting profiles of duplexes formed between Ts-AOs and RNA target. Conditions: 2.0 µM of each strand in 5 mM sodium phosphate buffer (35 mM NaCl, 50 µM Na_2-EDTA, pH 7.0).

Cell culture and transfection

H2K mdx mouse myoblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 20% fetal bovine serumn(FBS), 10% horse serum (HS) supplemented with 0.5% chicken embryo extract until reached 60–90% continuency. Prior to cell seeding, the 24-well plate was pre-treated with 50 mg mL^{-1} poly-D-lysine (Sigma) and 100 mg mL^{-1} Matrigel (Corning). Then, the myoblasts were seeded at 25 × 10^3 cells per well in DMEM 5% HS and incubated at 37 °C, 5% CO_2 for 48 hours for differentiation into myotubes.

For transfection, antisense oligonucleotides were complexed with Lipofectin (Life Technologies) at the ratio of 2 : 1 (Lipofectin : AO) with final concentrations as shown on Figures 2, 3 and Fig. S5 in a volume of 500 mL per well as per the manufacturer's instructions without removing the medium after 3 hours.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Twenty-four hours after transfection, the cells were collected for RNA extraction. Briefly, RNA was extracted using Direct-zolTM RNA MiniPrep Plus with TRI Reagent® (Zymo Research) as per the manufacturer's instructions. The dystrophin transcripts were then amplified by nested RT-PCR across exons 20–26 as cells were seeded and transfected with AOs as described.

4. DMD Ex23 skipping experiment

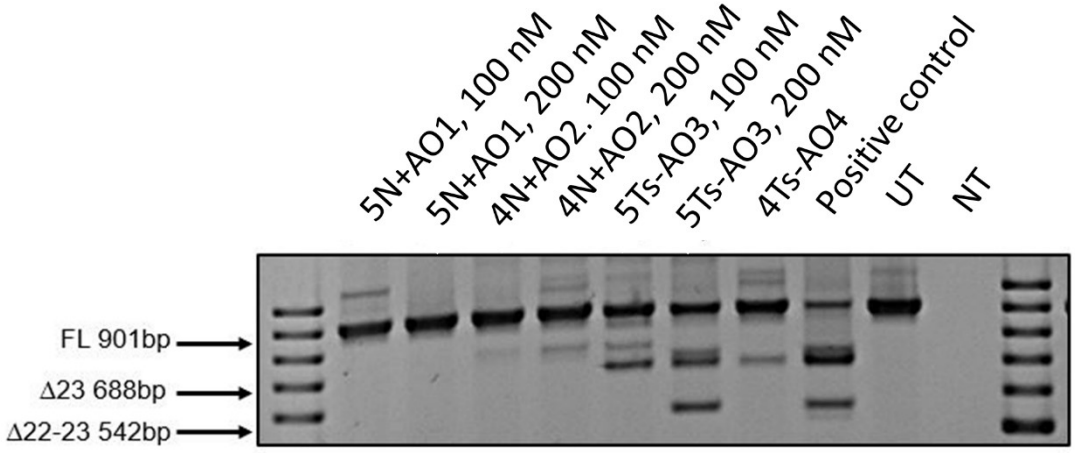

Figure S23. RT-PCR analysis of modified AOs for exon-23 skipping in mdx mouse myotubes. 901 bp: full length; 688 bp: exon-23 skipped product; 542 bp: dual exon 22/23 skipped product; UT: untreated; NT: negative control; fully modified 2′-OMePS-RNA: positive control. AOs were tested at two concentrations: 100 nM and 200 nM. **5Ts-AO3** induced exon skipping at both concentrations tested. No skipping was observed for **5N+AO1** and **4N+AO2**.

Table S2. Results of exon-23 skipping and dual exon-22/23 skipping of modified AOs at different concentrations based on Figures 3 and 4.

	Exon-23 skipping (%)				**Dual exon-22/23 skipping (%)**			
	12.5 nM	**25 nM**	**50 nM**	**100 nM**	**12.5 nM**	**25 nM**	**50 nM**	**100 nM**
Positive control	50	50	70	65	15	15	20	28
5N+AO1	-	8	27	33	-	-	16	15
5N+ all PS-AO9	32	22	47	61	-	-	16	7
5Ts-AO3	12	31	51	44	7	10	5	28
7Ts-AO6	8	14	34	41	-	6	11	6
5Ts-5PS-AO8	37	47	44	78	-	21	26	-
5Ts- all PS-AO10	40	40	48	44	5	21	17	25

"-" not detected.

References

1. Y. Su, P. J. B. Edwards, D. A. Stetsenko and V. V. Filichev, *ChemBioChem*, 2020, **21**, 2455-2466.
2. L. F. Tietze and T. Eicher, *Reactions and Synthesis in the Organic Chemistry Laboratory* Georg Thieme Verlag Stuttgart, New York, 1991.
3. D. V. Prokhorova, B. P. Chelobanov, E. A. Burakova, A. A. Fokina and D. A. Stetsenko, *Russ. J. Bioorgan. Chem.*, 2017, **43**, 38-42.
4. G. G. Hazen, F. W. Bollinger, F. E. Roberts, W. K. Russ, J. J. Seman and S. Staskiewicz, *Organic Synthesis, Vol 73*, 1996, **73**, 144-151.
5. J. L. Mergny and L. Lacroix, *Oligonucleotides*, 2003, **13**, 515-537.