Electronic Supplementary Material (ESI) for Analyst.

Supporting Information

Establishing Stereochemical Comparability in Phosphorothioate Oligonucleotides with Nuclease P1 Digestion Coupled with LCMS Analysis

Zifan Li*, Fei Tong, Li Xiao, Nicholas R. Larson, Xuan Zhou, Yueheng Zhang, Jonas P. Immel-Brown, George M. Bou-Assaf*

Pharmaceutical Operations & Technology, Biogen, 225 Binney Street, Cambridge, MA 02142, USA

*Corresponding authors

Email: zifan.li@biogen.com; george.bou-assaf@biogen.com

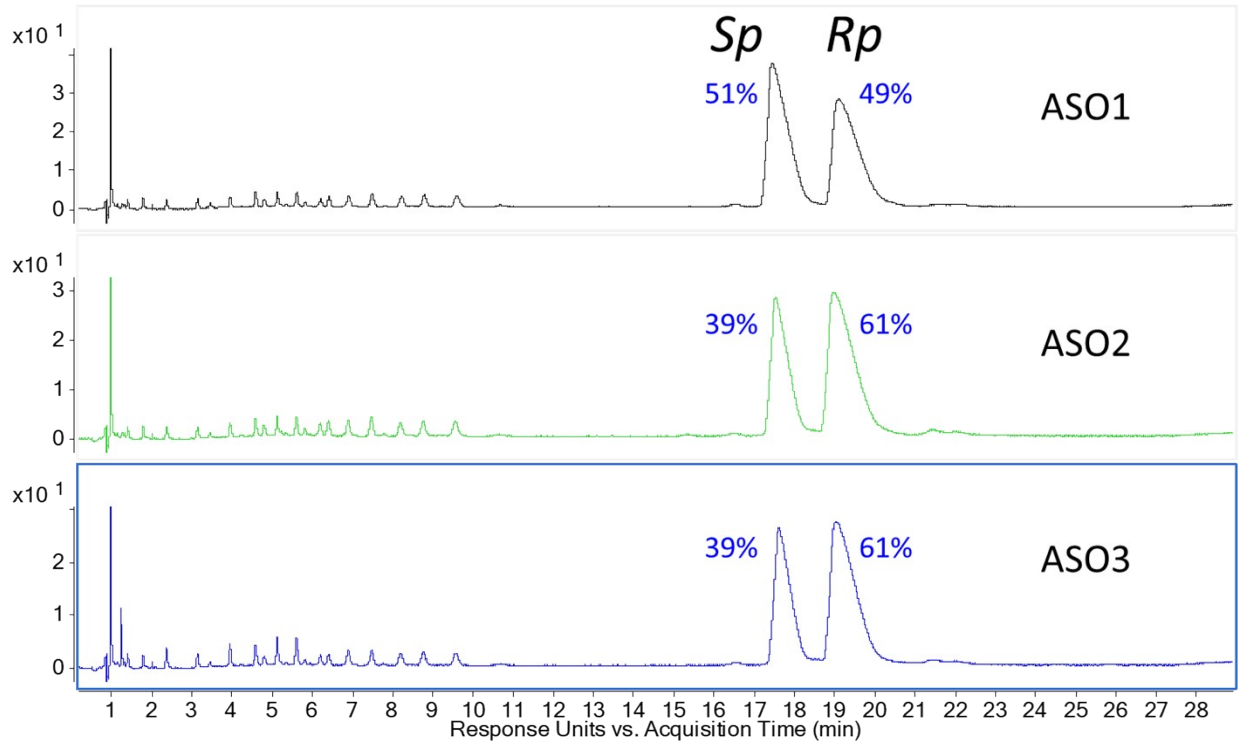

Fig. S1 UV chromatograms (260 nm) of crude ASOs (DMT-on)

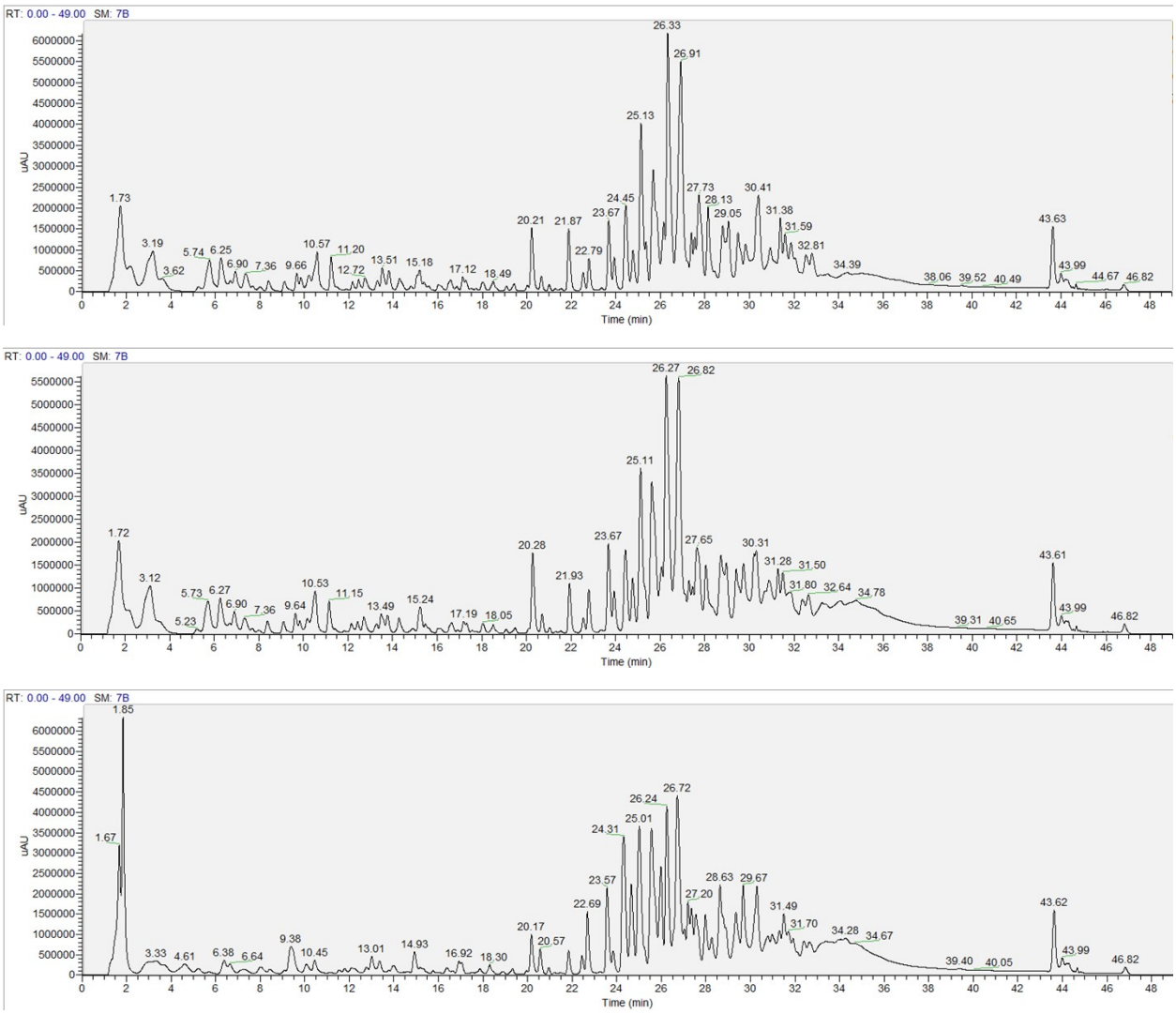

Fig. S2 UV chromatogram (260 nm) after 1 day of NP1 digestion. Top: ASO1; Middle: ASO2; Bottom: ASO3.

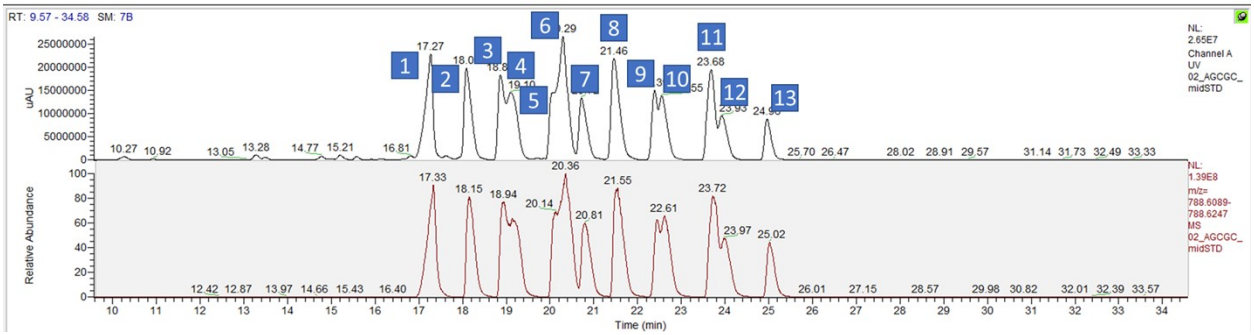

Fig. S3 Chromatograms of AG^{Me}CG^{Me}C standard (all PS modified). Top: UV at 260 nm; Bottom: EIC

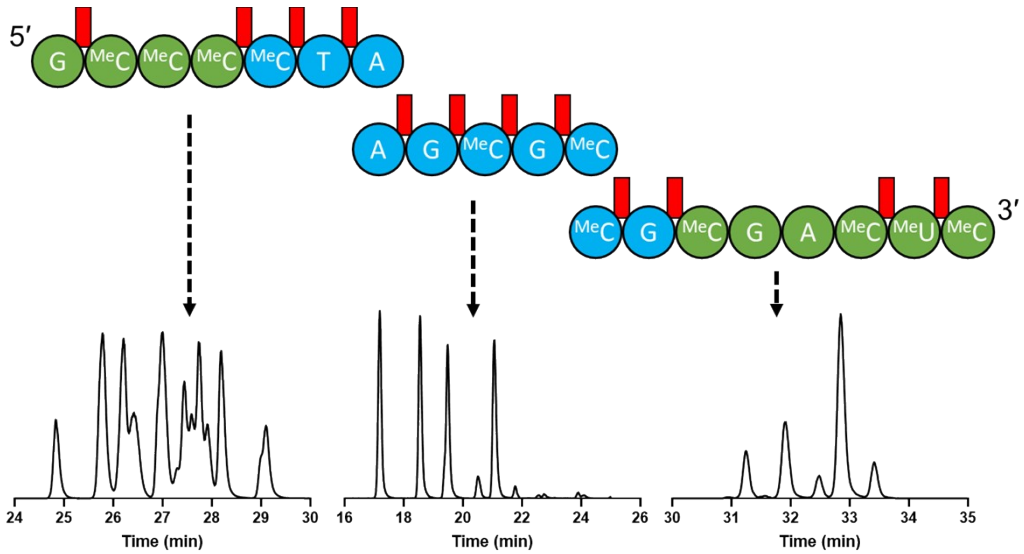

Fig. S4 Example of 3 characteristic fragments covering the entire ASO1 sequence and their EICs

1. Experimental Section

1.1. Oligonucleotide Synthesis

Syntheses of ASOs were performed using an AKTA oligopilot 100 solid-phase synthesizer equipped with a Nittophase HL Unylinker (Kinovate) solid support on a fixed height stainless-steel column (Φ 2 x 2.0 cm) at ambient temperature (~23 °C). The syntheses were conducted at 0.22 mmol scales based on the loading density of the DMT group. The synthetic process consists of detritylation, coupling, and sulfurization/oxidation. The detritylation step was carried out using a 10% dichloroacetic acid in toluene as the deblock reagent with a typical reaction time (3.85 min for the 1st cycle, 2.75 min for cycles 2-16 and 3.00 min for the cycles 16-18). The coupling step was performed by delivery of the same volume of corresponding phosphoramidite (3.4 eq, 0.2 M in acetonitrile) and corresponding activator solution: ASO1: 1.0 M DCI (17 eq) in acetonitrile containing 0.1 M NMI; ASO2: Cycles 1-17: 1.0 M DCI (17 eq) in acetonitrile containing 0.1 M NMI, Cycle 18: 0.3 M BTT (8.5 eq) in acetonitrile; ASO3: 0.3 M BTT (8.5 eq) in acetonitrile. The coupling reagents were circulating the mixed solutions in the synthesis column. The total reaction time for a coupling step was 4.95 min. The sulfurization step was carried out by delivery of xanthane hydride (6 eq, 0.2 M in pyridine) followed by a 3 min sulfurization reagent circulation. The oxidation step was performed by delivery of oxidation reagent (3.0 eq 0.05 M iodine in pyridine/water = 90:10 (v:v)). After the synthetic sequence was completed, the phosphorus protecting group was removed by circulating a mixture of triethylamine and acetonitrile (1:1, (v/v)). Cleavage from the solid support and deprotection of the ASOs were completed in concentrated ammonium hydroxide at 55 °C for 11 h.

1.2. Crude UHPLC analysis

The crude sample was dried in Speedvac and resuspended in 0.01% TEA in water to make 0.1 mg/mL solution, which was then injected to Agilent 1290 UHPLC, using Waters ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm X 150 mm (Part#186002353). Mobile phase A was 5 mM TBuAA, 10% ACN, 1 µM EDTA. Mobile phase B was 5 mM TBuAA, 80% ACN, 1 µM EDTA. Flow rate was 0.45 mL/min. Column temperature was set at 60°C. Mobile phase B was increased from 54% to 57% over 22.5 minutes.

1.3. Hydrophobic interaction chromatography (HIC) purification and detritylation

Crude (DMT-on) ASO was diluted 1:9 into 888 mM ammonium sulfate and 50 mM Tris and then bound to Cytiva Phenyl Sepharose 6 Fast-Flow High Substitution resin (Product#17097303) packed to a 20.5 cm bed height, in a Millipore Vantage 1.1 cm ID chromatography column (Part#96100250). The resin-bound product was washed with 400 mM ammonium sulfate and 50 mM Tris, then removed from the resin using a solution of 40 mM ammonium sulfate and 50 mM Tris. The purified product was deprotected by titration to pH 3.4 using 50% acetic acid and incubation at 27 °C for 4 hours. The deprotected product was filtered through a 0.2 µm polyethersulfone membrane to remove precipitated DMT-OH. The product was desalted using Millipore Amicon Ultra-15 centrifugal filter units containing 3 kDa regenerated cellulose membranes (Part#UFC900308) to retain the product.

1.4. NP1 digestion

NP1 (New England Biolabs, 100000 U/mL) stock solution was diluted 100 times with MilliQ water. For every 2.5 nmol of full-length ASO (10~15 mg/mL in water), 1 U of NP1, 10x digestion buffer (New England Biolabs) and appropriate amount of MilliQ water was

added for digestion. The final full-length ASO concentration was kept at 0.3 mg/mL. The solution was incubated at 37°C for 1 day.

1.5. LCMS condition

Agilent 1290 infinity UHPLC coupling to Thermo Orbitrap Fusion mass analyzer and Waters ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm X 100 mm (Part#186002352) were used. Column temperature was kept at 30°C during the run. Mobile phase A was 10 mM TEAA in water, pH 7.0 and mobile phase B was LCMS grade methanol. Gradient starts from 10%B and increases 0.5%B per min. Flow rate was 0.3 mL/min.

1.6. LCMS analysis

Thermo Xcalibur Qual Browser was used for data analysis. The theoretical masses of all possible digested fragments were calculated and matched with the observed masses. The monoisotopic m/z of the selected fragment was used to generate its EIC, with mass tolerance set at 20 ppm and Boxcar smoothing (7 points) applied.

Abbreviations:

ASO: Antisense oligonucleotides

UV: Ultraviolet

NP1: Nuclease P1

DCI: 4,5-Dicyanoimidazole

NMI: N-methylimidazole

BTT: 5-(Benzylthio)-1H-tetrazole

DMT: Dimethoxytrityl

EIC: Extracted ion chromatogram

TBuAA: Tributylammonium acetate

TEAA: Triethylammonium acetate

Tris: Tris(hydroxymethyl)aminomethane

TEA: Triethylamine

EDTA: Ethylenediaminetetraacetic acid

UHPLC: Ultra-high performance liquid chromatography

LCMS: Liquid chromatography mass spectrometry