Orthogonal Characterization of PMO Sequence, Structure, and Aggregation through a Regulatory Lens

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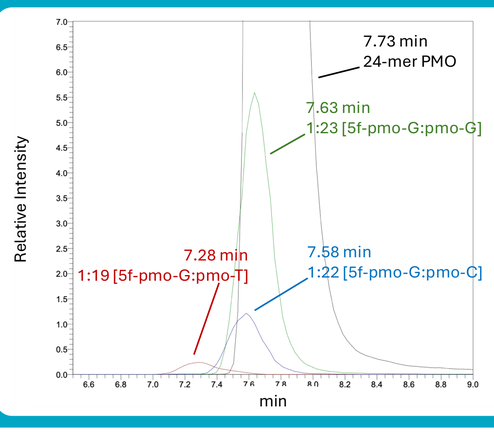
novatia

24mer Phosphorodiamidate Morpholino Oligonucleotide (PMO) Characterization Case Study

- **PMOs are a a growing class of next-generation therapeutics due to their high stability and low toxicity.**
- **As with other complex APIs, regulatory agencies will require proper methods to evaluate impurities, aggregation, and structure of innovator products and eventually demonstrate sameness for generic products.**
- ***Using Novatia's experience in providing complex API sameness characterization, we have outlined an approach to evaluate and characterize a 24mer PMO sequence***

Impurity Profiling by LC-HRMS

PMO Forced Degradation HRMS-LCMS XIC



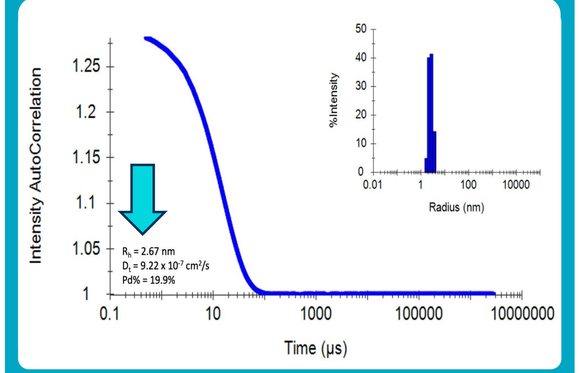
Identified Species and XIC Areas

RT	Monoisotopic Mass (Da)	Identity	XIC area
7.73	8174.813	24-mer PMO	60436162
7.63	7844.700	1:23 [5f-pmo-G:pmo-G]	3674179
7.58	7489.583	1:22 [5f-pmo-G:pmo-C]	863699
7.28	6464.247	1:19 [5f-pmo-G:pmo-T]	189857

PMO impurities can be separated, identified, and quantified by LC-HRMS

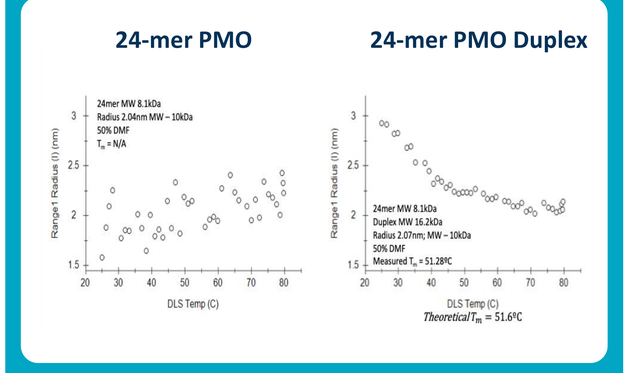
Aggregation Characterization by DLS

DLS Autocorrelation



DLS yields diffusion coefficient (D_t) and hydrodynamic radius (R_h)

Melting Temperature for PMO Duplex

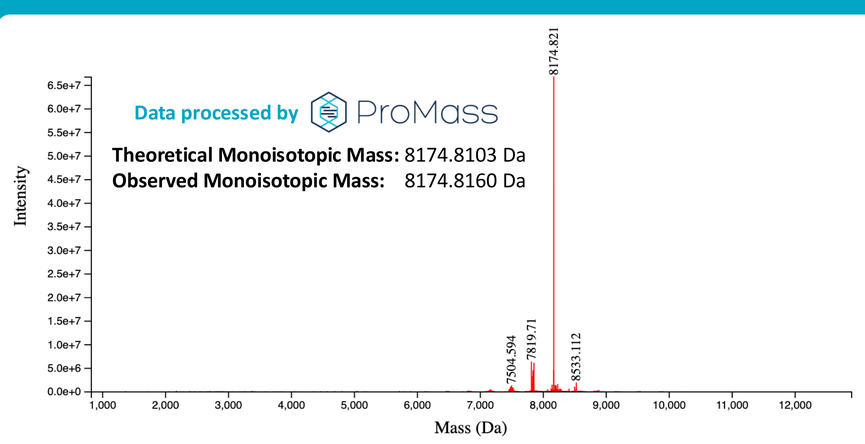


DLS temperature dependence yields a change in R_h with increasing temperature

Orthogonal methods confirm expected size of 24-mer PMO, no aggregates present

Sequence Confirmation by HRMS and MS/MS

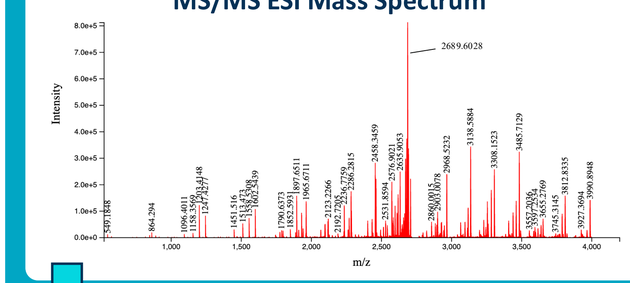
Intact Deconvoluted Mass Spectrum



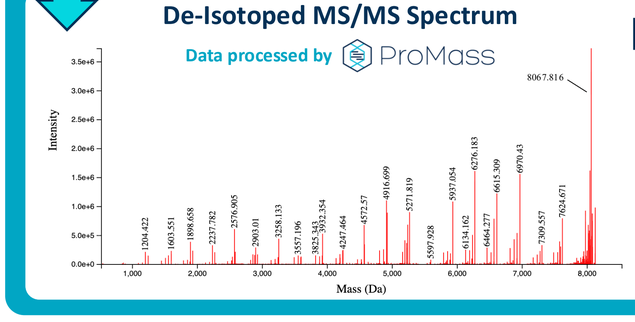
HRMS confirms monoisotopic mass of the intact sequence with a mass accuracy of 0.7 ppm

HRMS provides highest confidence for intact sequence identity of PMO

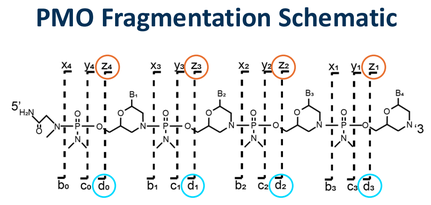
MS/MS ESI Mass Spectrum



De-Isotoped MS/MS Spectrum



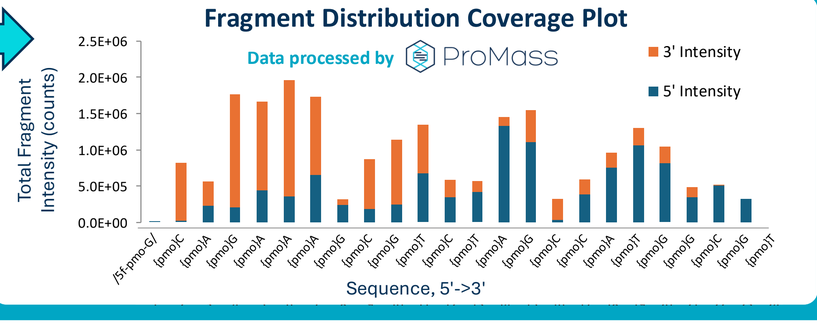
PMO Fragmentation Schematic



5' d ions and 3' z ions are primarily formed via ion trap collisional activation in negative mode

MS/MS Sequencing with Novatia's ProMass OligoSeq tool matches observed MS/MS species to theoretical PMO-specific fragments to provide high confidence sequence confirmation.

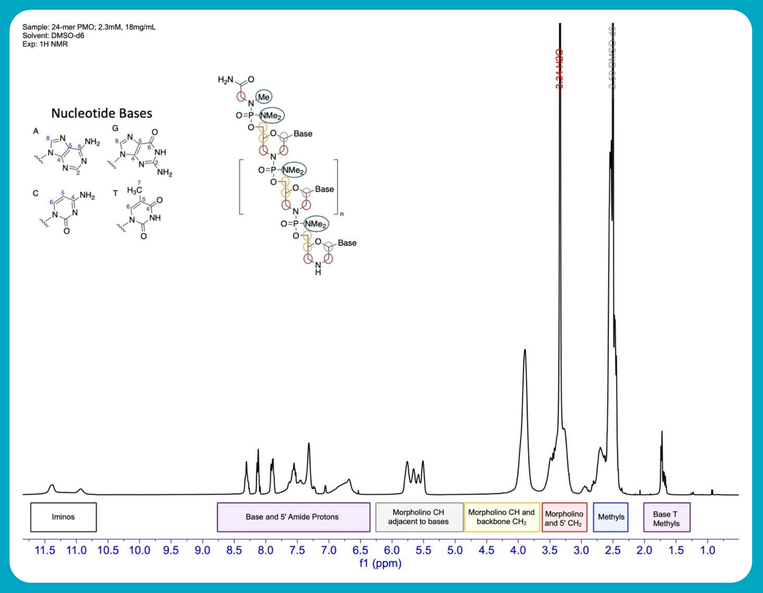
Fragment Distribution Coverage Plot



Wang, M., O'Day, B., Michaels, B., Juravj, J., Cai, B.Z. & Wei, T. Sequencing of Phosphorodiamidate Morpholino Oligomers by Hydrophilic Interaction Chromatography Coupled to Tandem Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* (2024). https://pubs.acs.org/doi/10.1021/jasms.4c00281

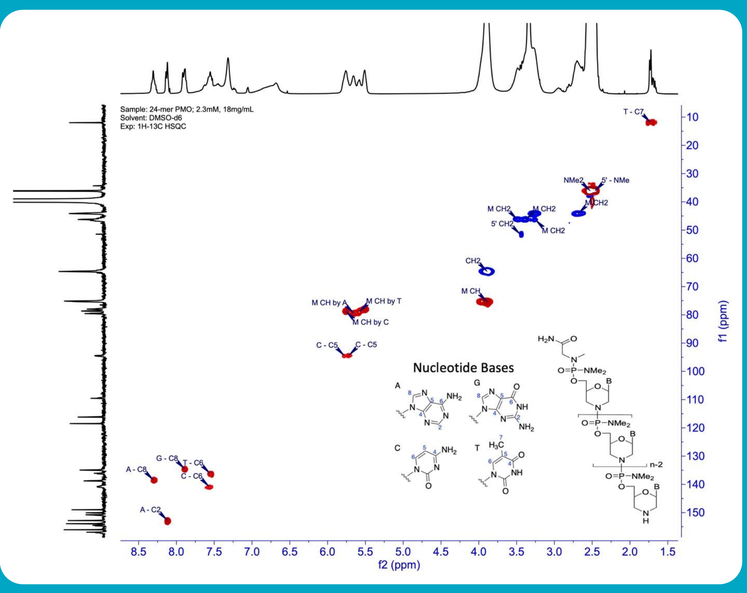
Solution Structure Fingerprinting by NMR

^{1}H Spectrum

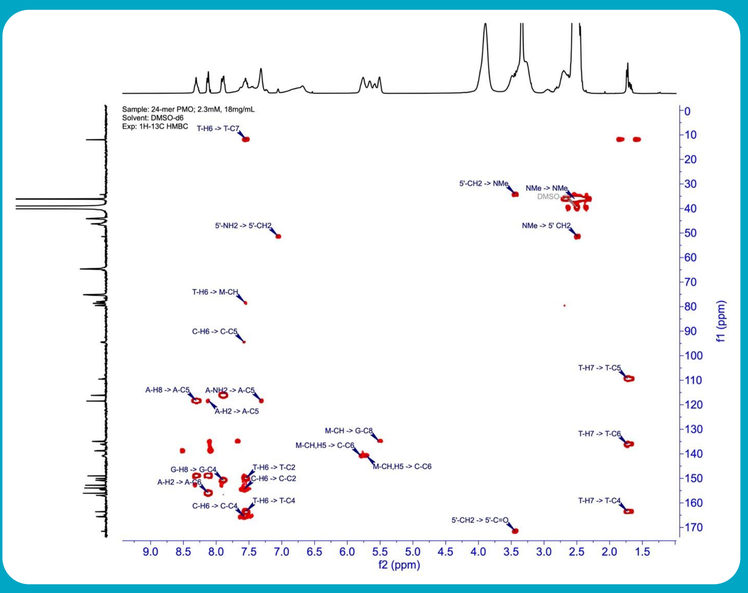


1D spectrum confirms characteristic backbone and bases of PMO

^{1}H-^{13}C HSQC Spectrum



^{1}H-^{13}C HMBC Spectrum



2D HMBC and HSQC spectra in combination provide a more detailed view of PMO structure and confirm which bases are present

NMR can provide a unique structure fingerprint that can be used for advanced statistical comparisons to evaluate sameness across production lots, formulations, and innovator vs. generic products

Conclusions

As PMO therapeutics grow in popularity, regulatory requirements for approval of innovator and generic drug products will expand. Novatia's experience navigating the regulatory landscape for other complex APIs positions us to offer comprehensive analytical services and help clients interpret and respond to regulatory needs in this rapidly evolving field:

1) **LC-HRMS methods can identify and quantify PMO-related impurities.**
2) **DLS can evaluate general aggregation and presence of duplex if relevant.**
3) **MS/MS characterization provides high-confidence detailed sequence confirmation.**
4) **NMR provides high-resolution data uniquely suited for statistical comparison across samples.**