18th Symposium on the Practical Applications of Mass Spectrometry in the Biotechnology Industry
Friday, September 24, 2021

Analysis of Therapeutic Oligonucleotides by HRMS

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Roche

Oligo Therapeutics over time

VITRAVENE 250 MG
GANCICLOVIR SODIUM CAPSULES
Infectious Diseases
(Cytomegalovirus retinitis)

MACUGEN®
PEGAPTANIB SODIUM INJECTION
Ophthalmology
(AMD)

DEFITELIO®
(defibrotide sodium) injection 80 mg/mL
Cardio/Metabolism
(veno-occlusive disease)

KYNAMRO®
(mipomersen sodium) injection
200mg/ml
Cardio/Metabolism
(Familial hypercholesterolemia)

EXONDYS 51™
(eteplirsen) Injection
Neuroscience
(DMD)

SPINRAZA®
(nusinersen) injection 12 mg/5 mL
Neuroscience
(SMA)

HEPLISAV-B®
Hepatitis B Vaccine (Recombinant), Adjuvanted
Infectious
Diseases
(Hepatits B)

onpattro™
(patisiran) lipid complex injection
Neuroscience
(transthyretin amyloidosis)

Tegsedi®
(inotersen) injection 284 mg/1.5 mL
Neuroscience
(transthyretin amyloidosis)

waylivra®▼
(volanesorsen) injection 285 mg/1.5 mL
Cardio/Metabolism
(Familial chylomicronaemia)

GIVLAARI™
(givosiran) injection for subcutaneous use 189 mg/mL
Cardio/Metabolism
(acute hepatic porphyria)

VYONDYS 53
(golodirsen) Injection
Neuroscience
(DMD)

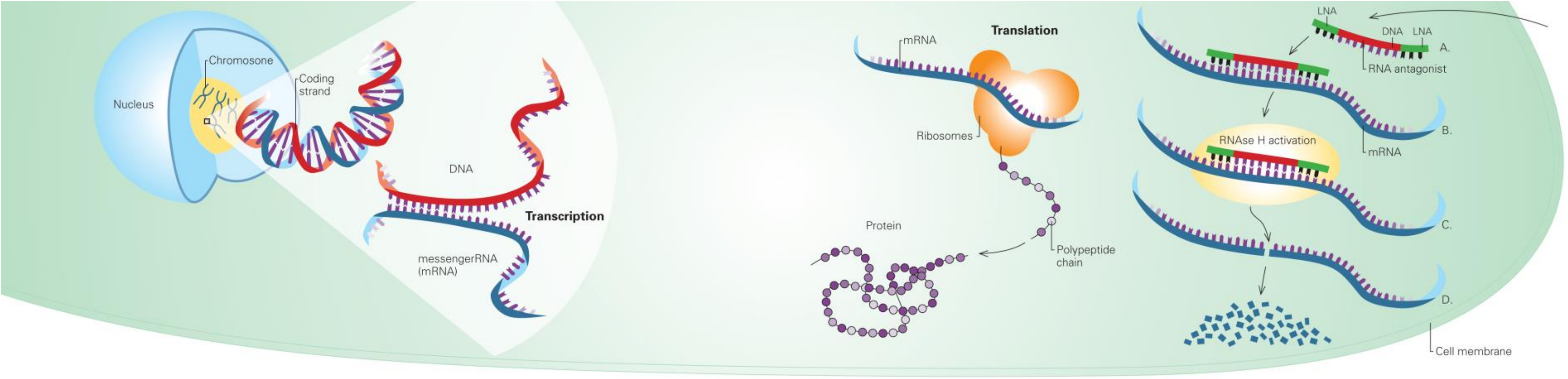
Viltepso
(viltolarsen) injection
Neuroscience
(DMD)

OXLUMO
(lumasiran) for injection 94.5 mg/0.5 mL
Primary Hyperoxaluria type 1
(Kidney failure)

Leqvio
Hypercholesterolaemia
(PCSK9)

1998 1999 2000 2001 2002 2003 2004 2005 2006 2007 2008 2009 2010 2011 2012 2013 2014 2015 2016 2017 2018 2019 2020

Antisense Oligonucleotides - Mode of Action

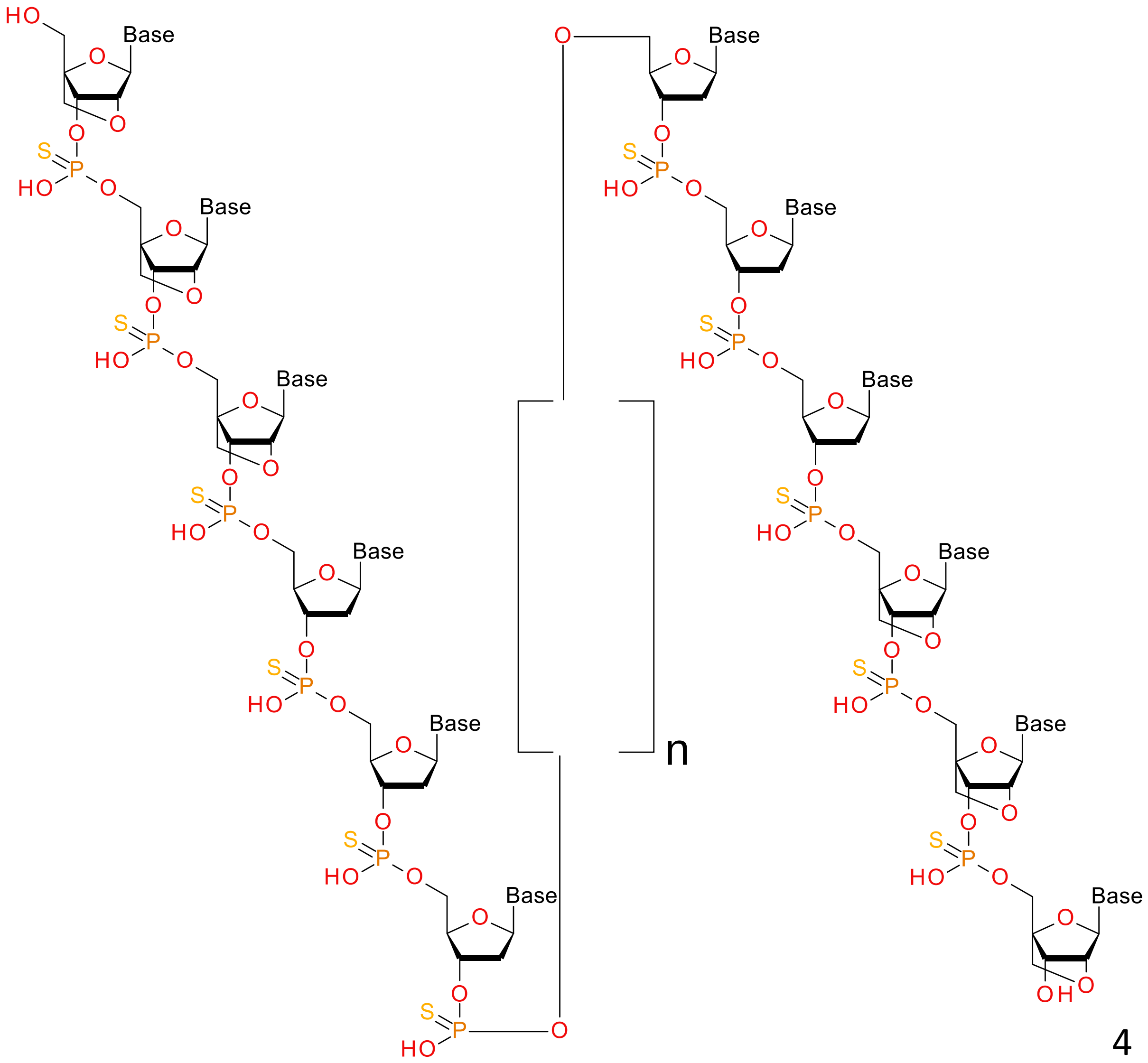


A. Oligonucleotides circulating in the blood are taken up by the cell
B. Oligonucleotide binds to its complementary sequence in the target mRNA associated with disease. Binding is potent and specific with no off-target effects
C. Cytoplasmic enzyme (RNAse H) activated which cleaves the target mRNA
D. mRNA translation is arrested and synthesis of disease-related protein prevented

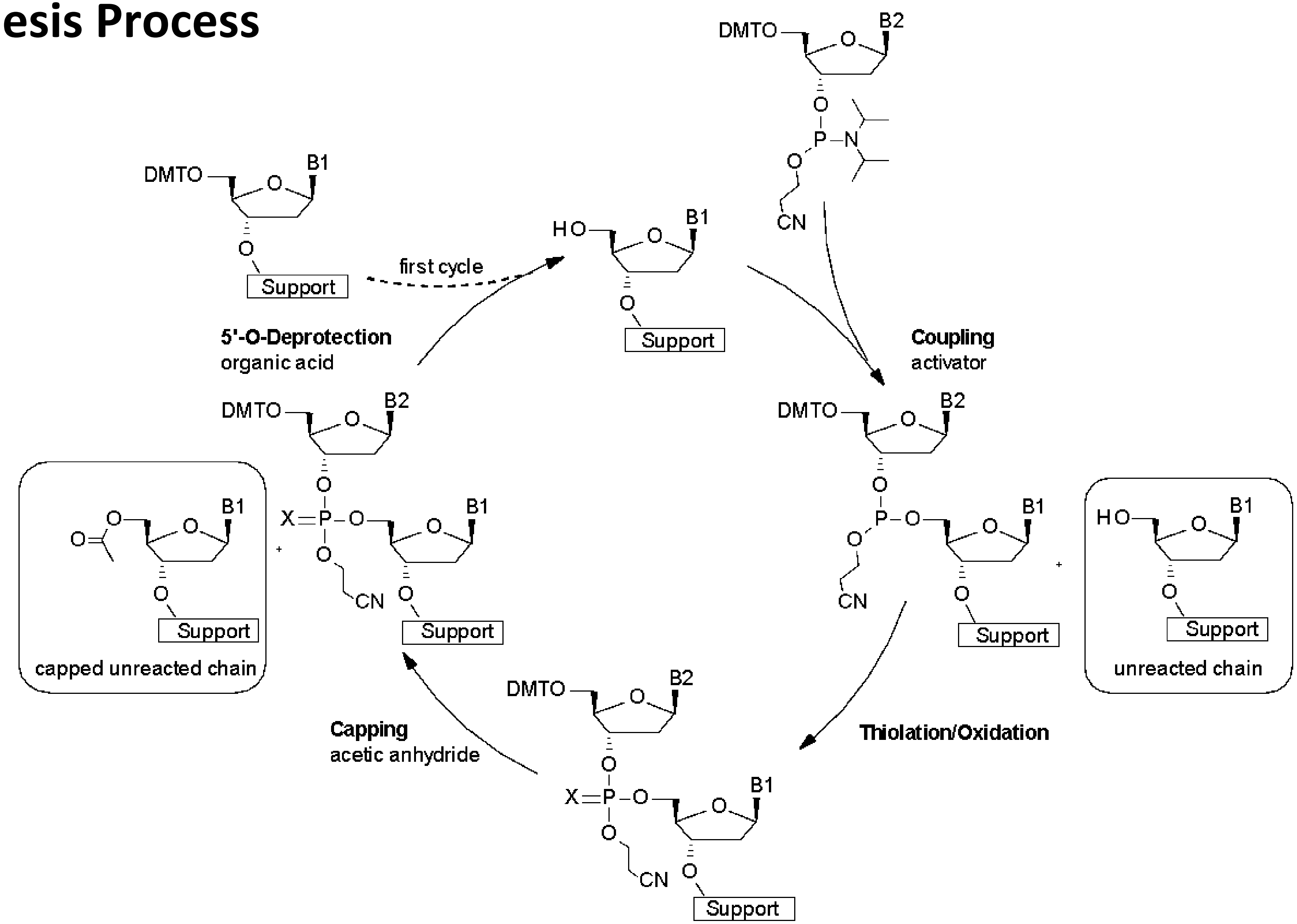
Slide from C. Steiner, Method Development for LNAs, May 2017

Antisense Oligonucleotides – Molecular Structure

- Approx. 20 nucleotides
- Nucleobase sequence for complementary binding to the target
- Backbone: Thioate modification improves stability towards enzymes
- Sugar modification: locked nucleic acid improves stability against nucleases and increases target affinity
- Other 2’-modifications i.e. 2’-Methoxy, 2’-MOE, 2’-Fluoro...
- Gapmer sturcture with DNA residues in the center is required for RNAase H mechanism



Synthesis Process



first cycle

5'-O-Deprotection
organic acid

Coupling
activator

Thiolation/Oxidation

Capping
acetic anhydride

Mass Spectrometry of Oligonucleotides

- High-resolution (qTOF, Orbitrap)
  - MS/MS for conformation of the correct sequence
    - for ID testing of incoming material
    - for identification of impurites
  - Identification of impurites by accurate mass
    - for process reasearch
    - for forced degradation studies
    - for troubleshooting

- Low-resolution (single Quad)
  - Routine analysis for process development
  - Routine analyses of ID by mass, determination of PO and other impurites

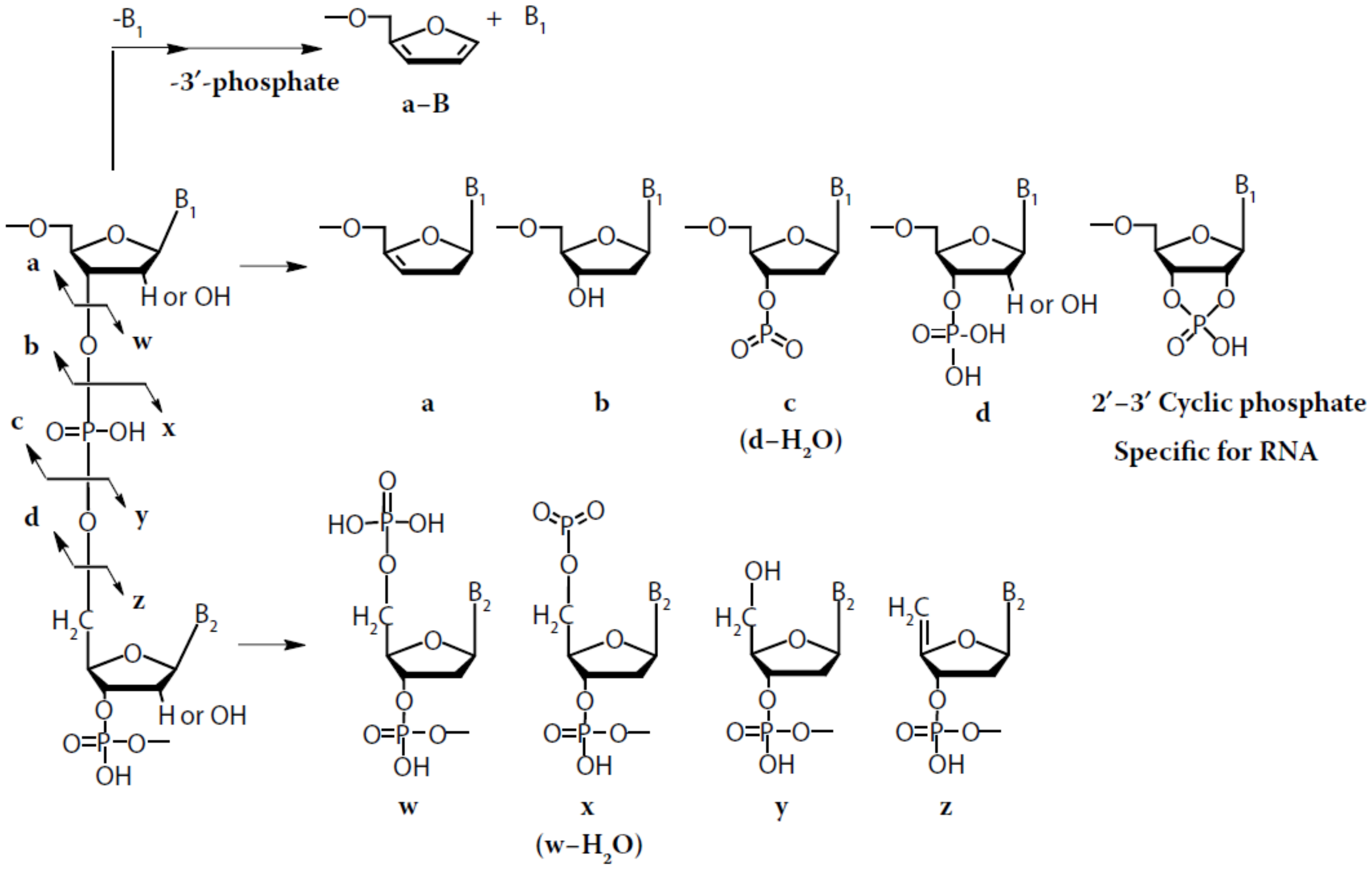
Agenda

- Identification of N-1 impurities by MS/MS
- Comparison of the impurity profile of in-process samples

Identification of N-1 impurities by MS/MS

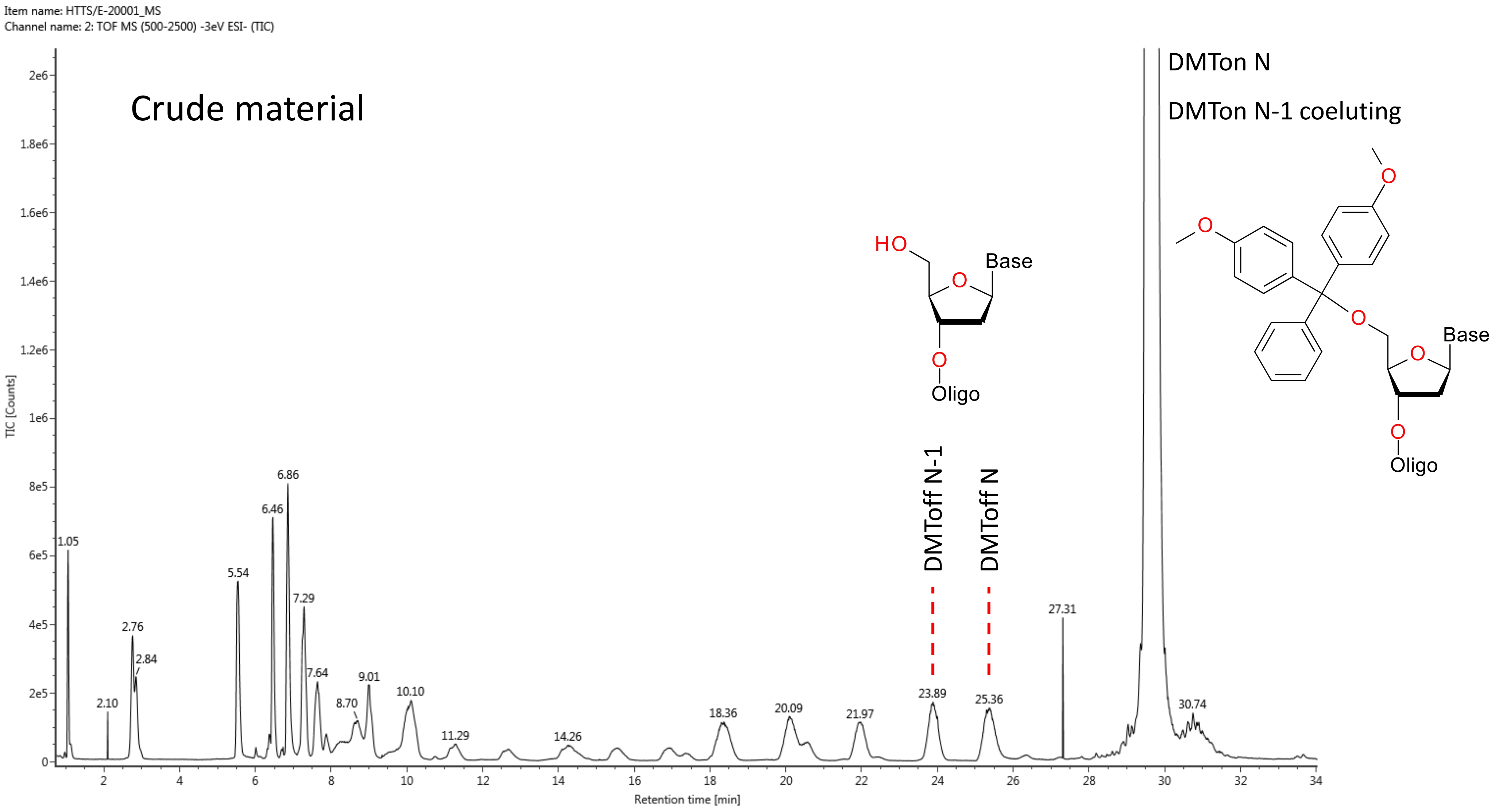
- Identification of N-1 impurities by MS/MS
  - Tominersen
    - 5-10-5 MOE-gapmer in developmet for Huntington’s disease
  - Problem:
    - Increased N-1 levels observed in several batches
    - Which coupling failed? N-first or N-last are most probable
    - First and last nucelotide are the same, therefore N-first and N-last have the same mass
    - Crude material (DMTon) and purified material (DMToff) was availabe
  - Solution:
    - MS/MS experiments of the N-1 impurity

Identification of N-1 impurities by MS/MS

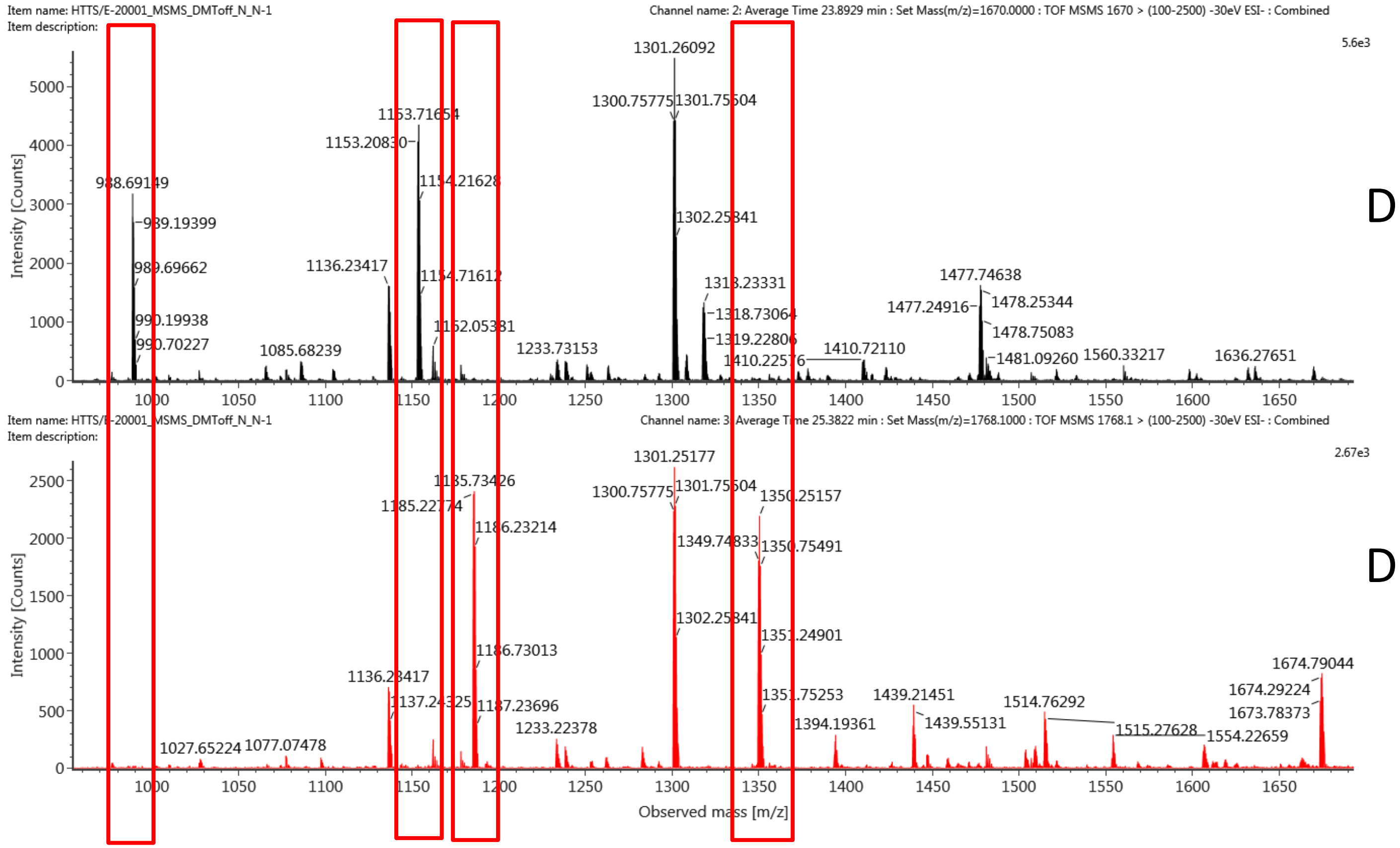


(from: Jose V. Bonnilla, Susan Srivatsa, Handbook of Analysis of Oligonucleotide and Related Products, CRC Press, Taylor & Francis Group, 2011)

Identification of N-1 impurities by MS/MS



Identification of N-1 impurities by MS/MS

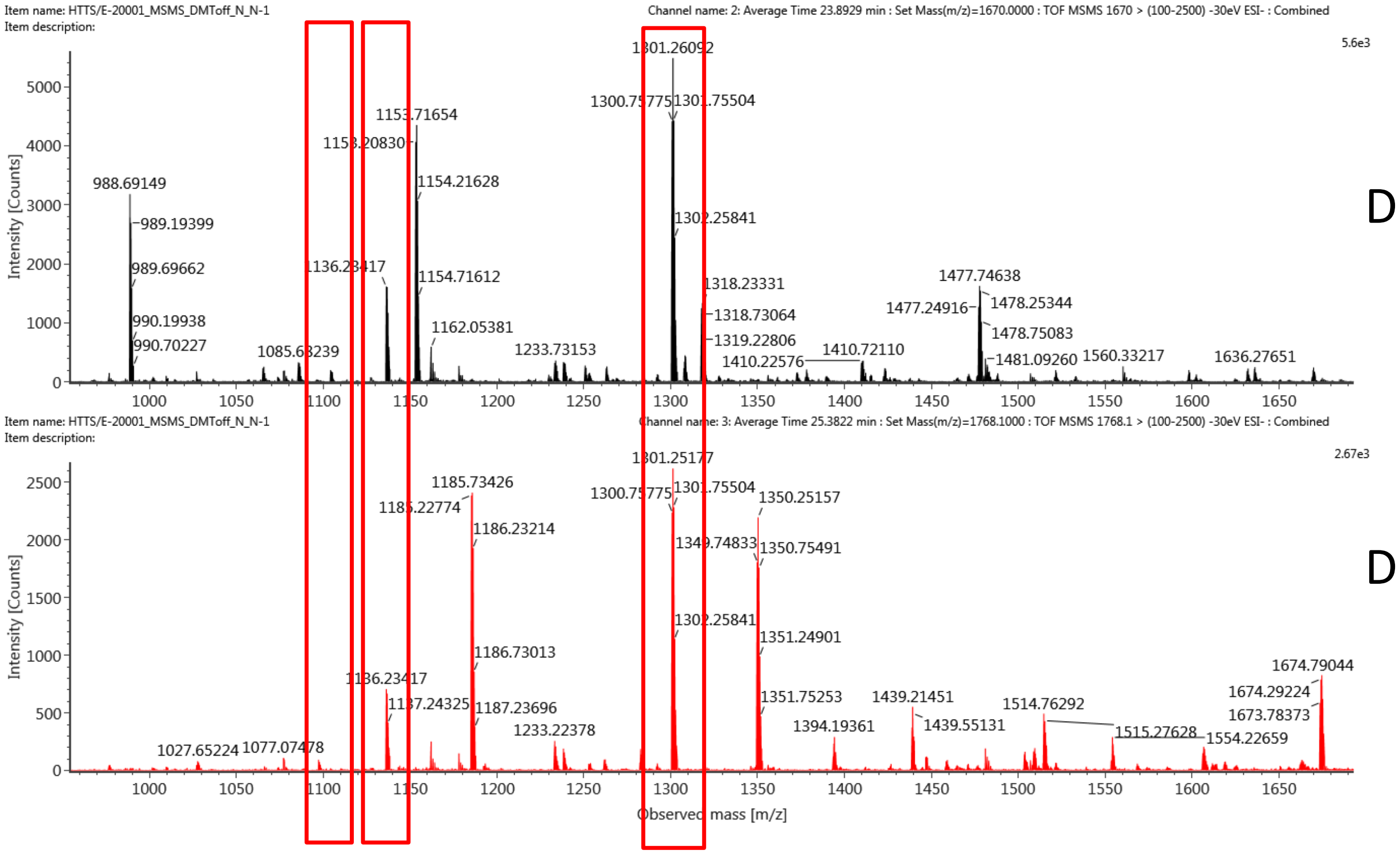


DMToff N-1

DMToff N

		5'-end	
$(a\text{-}B)_7$	m/z = 1185.24	`eEs.eTo.eEo.eAo.eGs.dTs.d`	only observed in N
$(a\text{-}B)_8$	m/z = 1349.77	`eEs.eTo.eEo.eAo.eGs.dTs.dAs.d`	only observed in N
$(a\text{-}B)_6$	m/z = 988.71	`eTo.eEo.eAo.eGs.dTs.d`	only observed in N-1
$(a\text{-}B)_7$	m/z = 1153.23	`eTo.eEo.eAo.eGs.dTs.dAs.d`	only observed in N-1

Identification of N-1 impurities by MS/MS



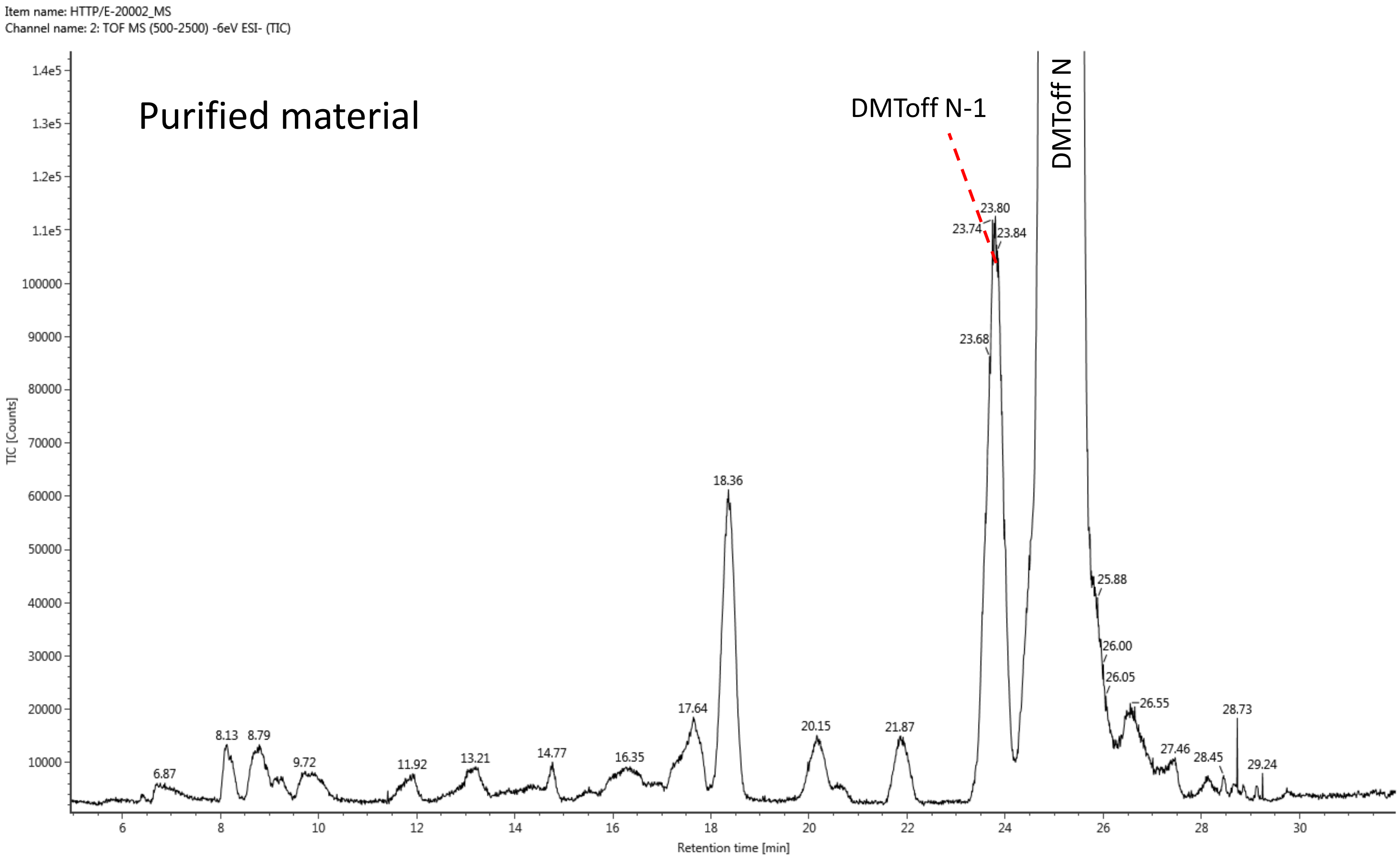
DMToff N-1

DMToff N

3’-end

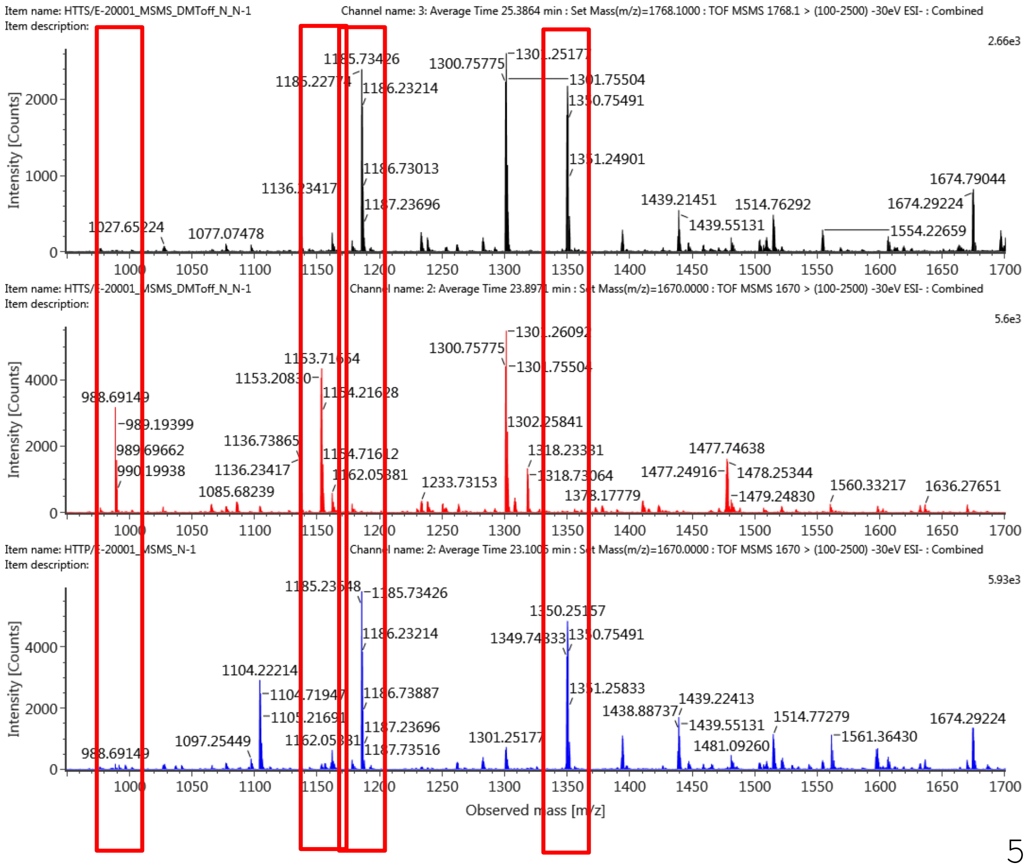
w6	m/z = 1136.25	s.dEs.eAo.eEo.eEo.eAs.eE	observed in N-1 & N
w7	m/z = 1300.77	s.dAs.dEs.eAo.eEo.eEo.eAs.eE	observed in N-1 & N
w5	m/z = 939.70	s.dEs.eAo.eEo.eEo.eA	not observed
w6	m/z = 1104.21	s.dAs.dEs.eAo.eEo.eEo.eA	not observed

Identification of N-1 impurities by MS/MS



Item name: HTTP/E-20002_MS
Channel name: 2: TOF MS (500-2500) -6eV ESI- (TIC)

Identification of N-1 impurities by MS/MS



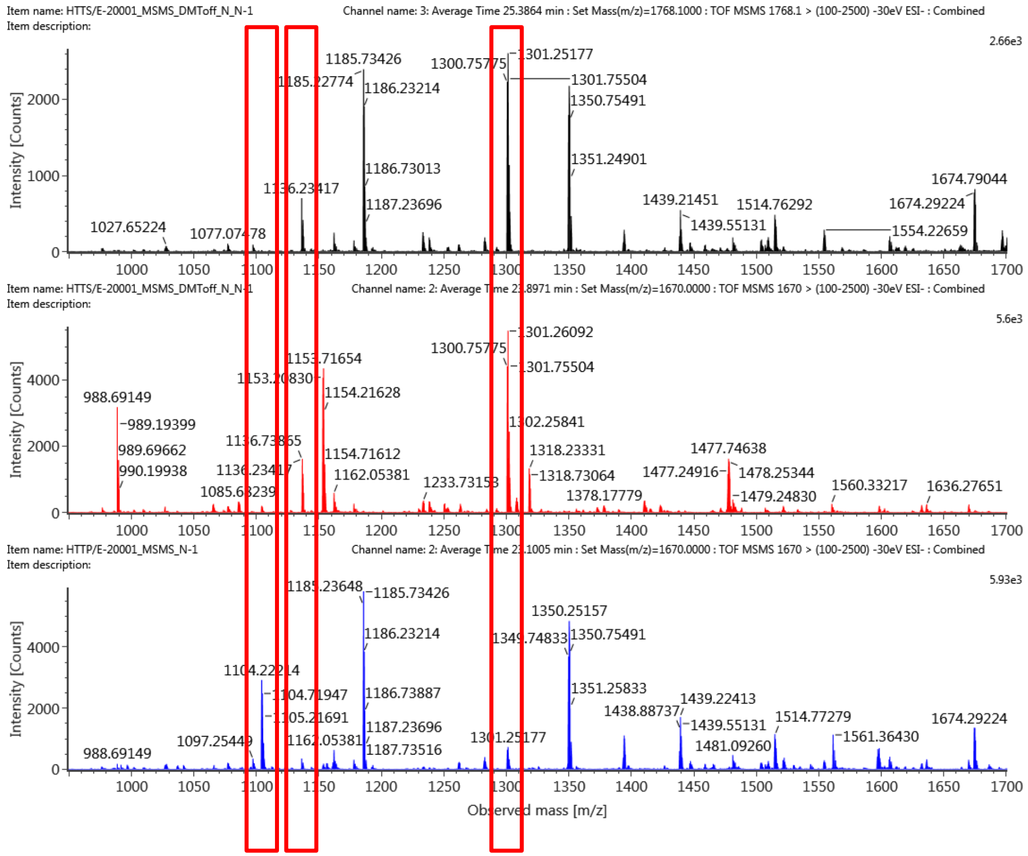
DMToff N from crude

DMToff N-1 from crude

DMToff N-1 from purified

		5′-end	
(a-B)$_7$	m/z = 1185.24	`eEs.eTo.eEo.eAo.eGs.dTs.d`	observed in N & N-1 from pure
(a-B)$_8$	m/z = 1349.77	`eEs.eTo.eEo.eAo.eGs.dTs.dAs.d`	observed in N & N-1 from pure
(a-B)$_6$	m/z = 988.71	`eTo.eEo.eAo.eGs.dTs.d`	not observed
(a-B)$_7$	m/z = 1153.23	`eTo.eEo.eAo.eGs.dTs.dAs.d`	not observed

Identification of N-1 impurities by MS/MS



DMToff N from crude

DMToff N-1 from crude

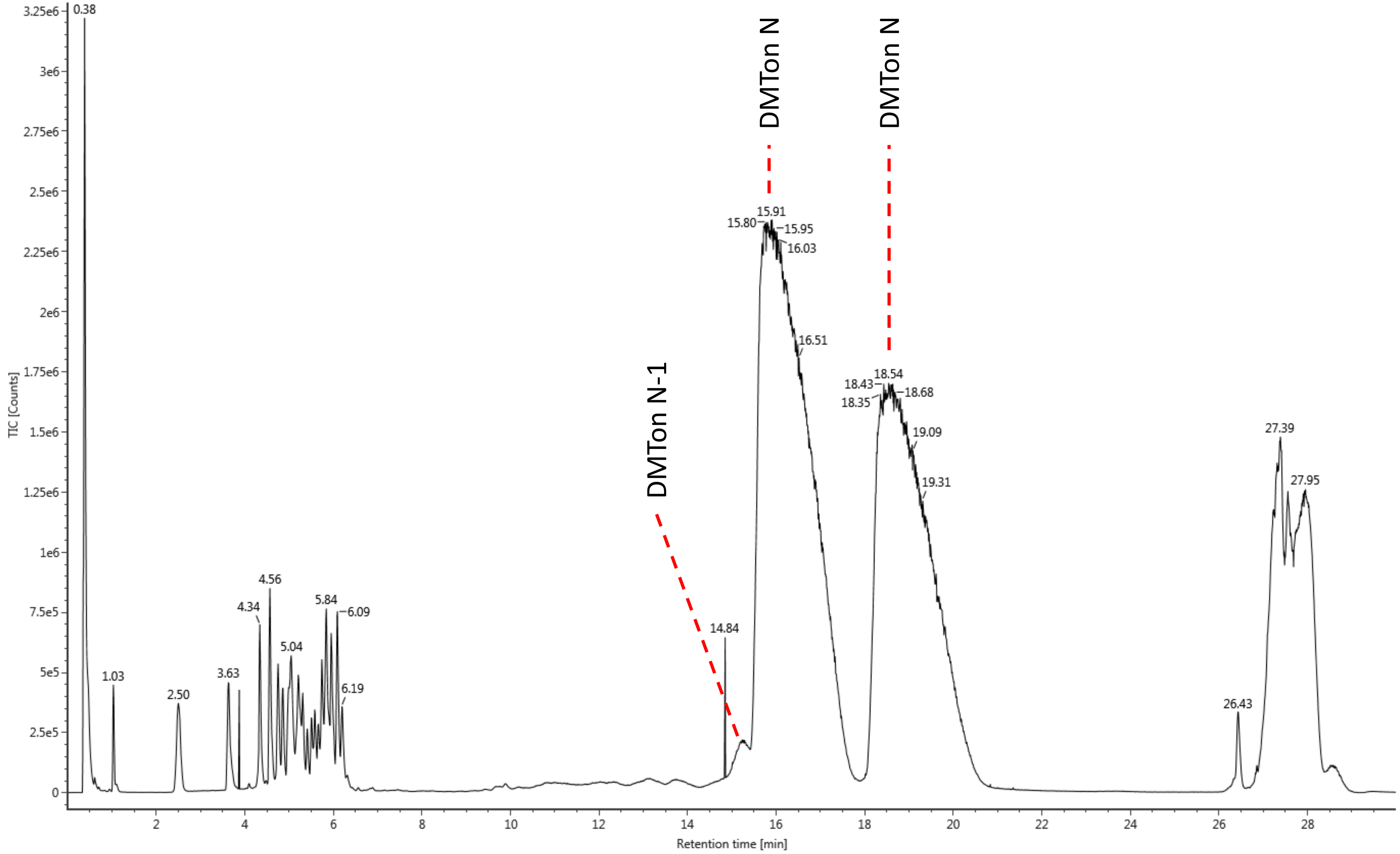
DMToff N-1 from purified

3’-end

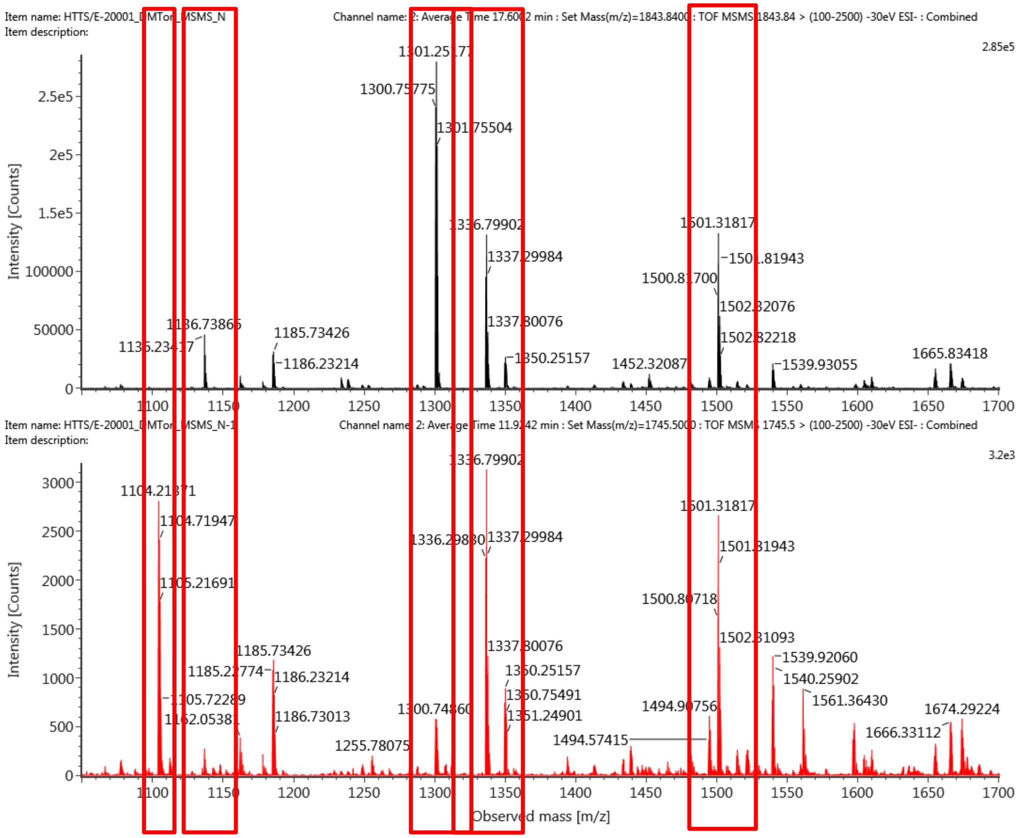
w_6	m/z = 1136.25	`s.dEs.eAo.eEo.eEo.eAs.eE`	weakly observed in N-1 from pure
w_7	m/z = 1300.77	`s.dAs.dEs.eAo.eEo.eEo.eAs.eE`	weakly observed in N-1 from pure
w_6	m/z = 1104.21	`s.dAs.dEs.eAo.eEo.eEo.eA`	observed in N-1 from pure

Identification of N-1 impurities by MS/MS

Item name: HTTS/E-20001_DMTon_MS
Channel name: 2: TOF MS (500-2500) -6eV ESI- (TIC)



Identification of N-1 impurities by MS/MS



DMT-5′-end

$(a\text{-}B)_7$	m/z = 1336.30	`eEs.eTo.eEo.eAo.eGs.dTs.d`	observed in N & N-1
$(a\text{-}B)_8$	m/z = 1500.82	`eEs.eTo.eEo.eAo.eGs.dTs.dAs.d`	observed in N & N-1
$(a\text{-}B)_6$	m/z = 1139.76	`eTo.eEo.eAo.eGs.dTs.d`	not observed
$(a\text{-}B)_7$	m/z = 1304.28	`eTo.eEo.eAo.eGs.dTs.dAs.d`	not observed

3′-end

w_6	m/z = 1104.21	`s.dAs.dEs.eAo.eEo.eEo.eA`	observed in N-1
w_6	m/z = 1136.25	`s.dEs.eAo.eEo.eEo.eAs.eE`	observed in N
w_7	m/z = 1300.77	`s.dAs.dEs.eAo.eEo.eEo.eAs.eE`	observed in N

Identification of N-1 impurities by MS/MS

- Conclusion
  - Crude material contains:
    - N-first DMTon
    - N-last DMToff

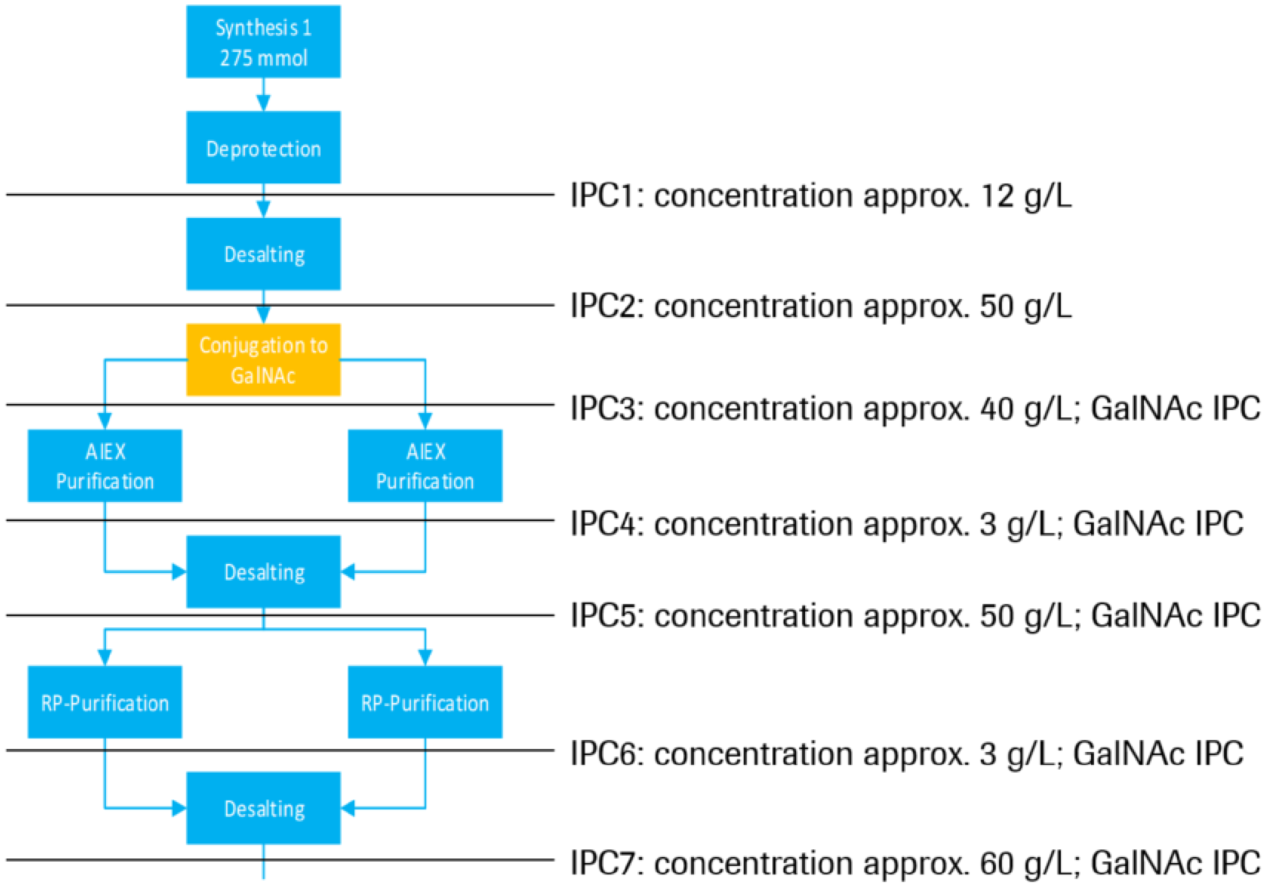
  - Purified material dominantly contains
    - N-first DMToff

Comparison of the impurity profile of in-process samples

- Comparison of the impurity profile of in-process samples
  - HBV-LNA
    - GalNAc conjugated LNA-15mer
  - Problem:
    - One out of three development batches had poor yield
    - In-process samples were taken at different stages of the process

  - Solution:
    - Impurity profile of in-process smaples was analyzed

Comparison of the impurity profile of in-process samples



IPC1: concentration approx. 12 g/L

IPC2: concentration approx. 50 g/L

IPC3: concentration approx. 40 g/L; GalNAc IPC

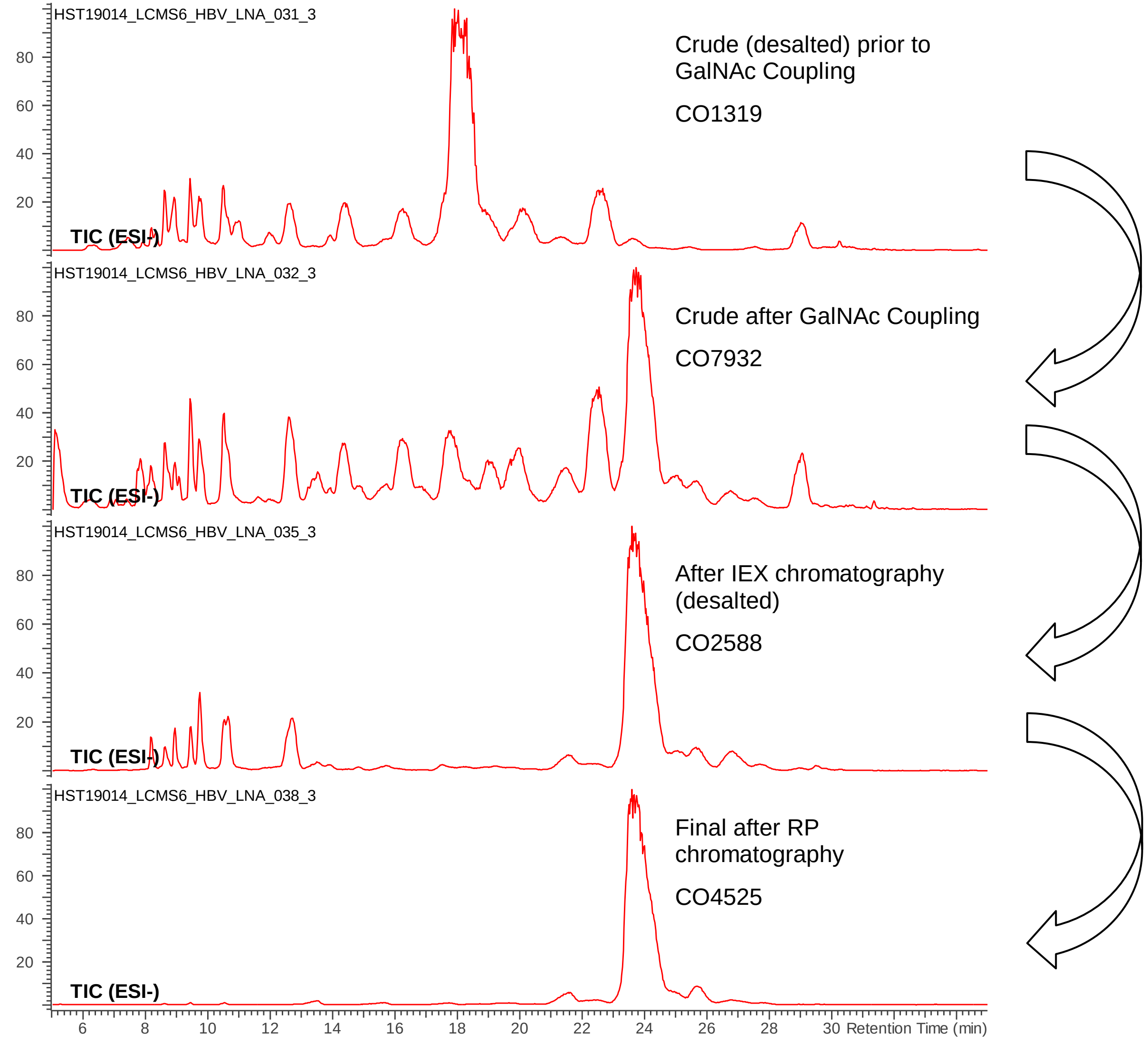
IPC4: concentration approx. 3 g/L; GalNAc IPC

IPC5: concentration approx. 50 g/L; GalNAc IPC

IPC6: concentration approx. 3 g/L; GalNAc IPC

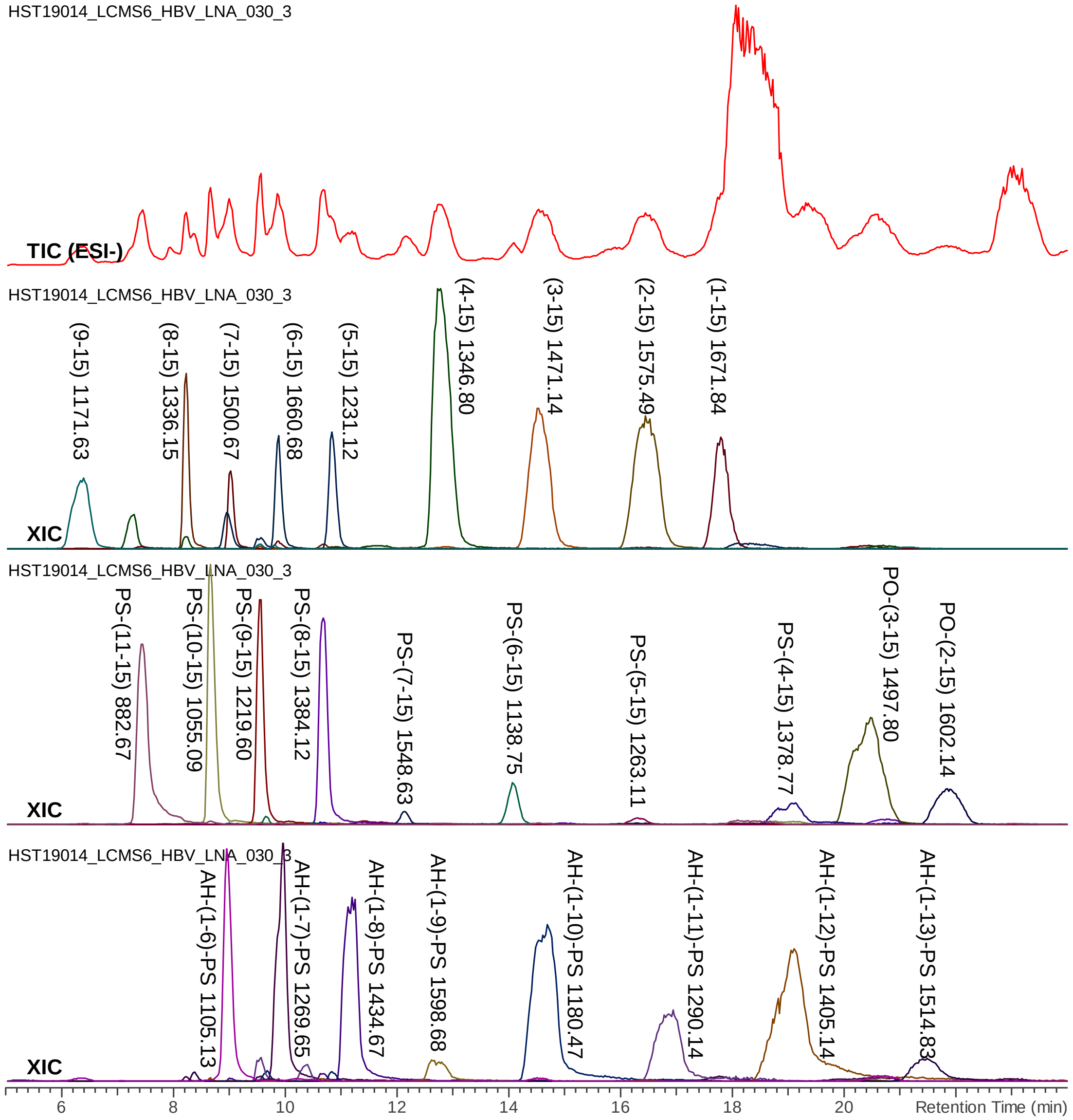
IPC7: concentration approx. 60 g/L; GalNAc IPC

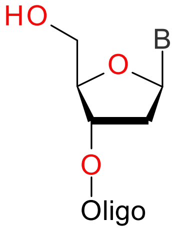
Comparison of the impurity profile of in-process samples



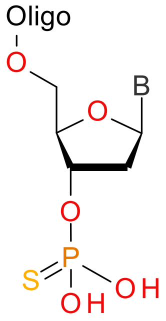
- Coupling of GalNAc Cluster results in a new main peak, impurity profile gets more complex
- Anion exchange chromatography depletes many impurities, but interestingly not some of the early eluting ones
- Reversed phase chromatography gives a very pure end product

Comparison of the impurity profile of in-process samples

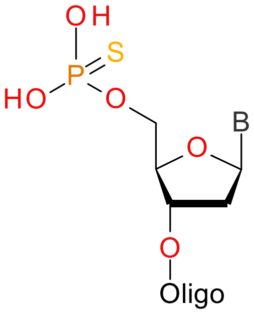




(x-end)



(1-x).s



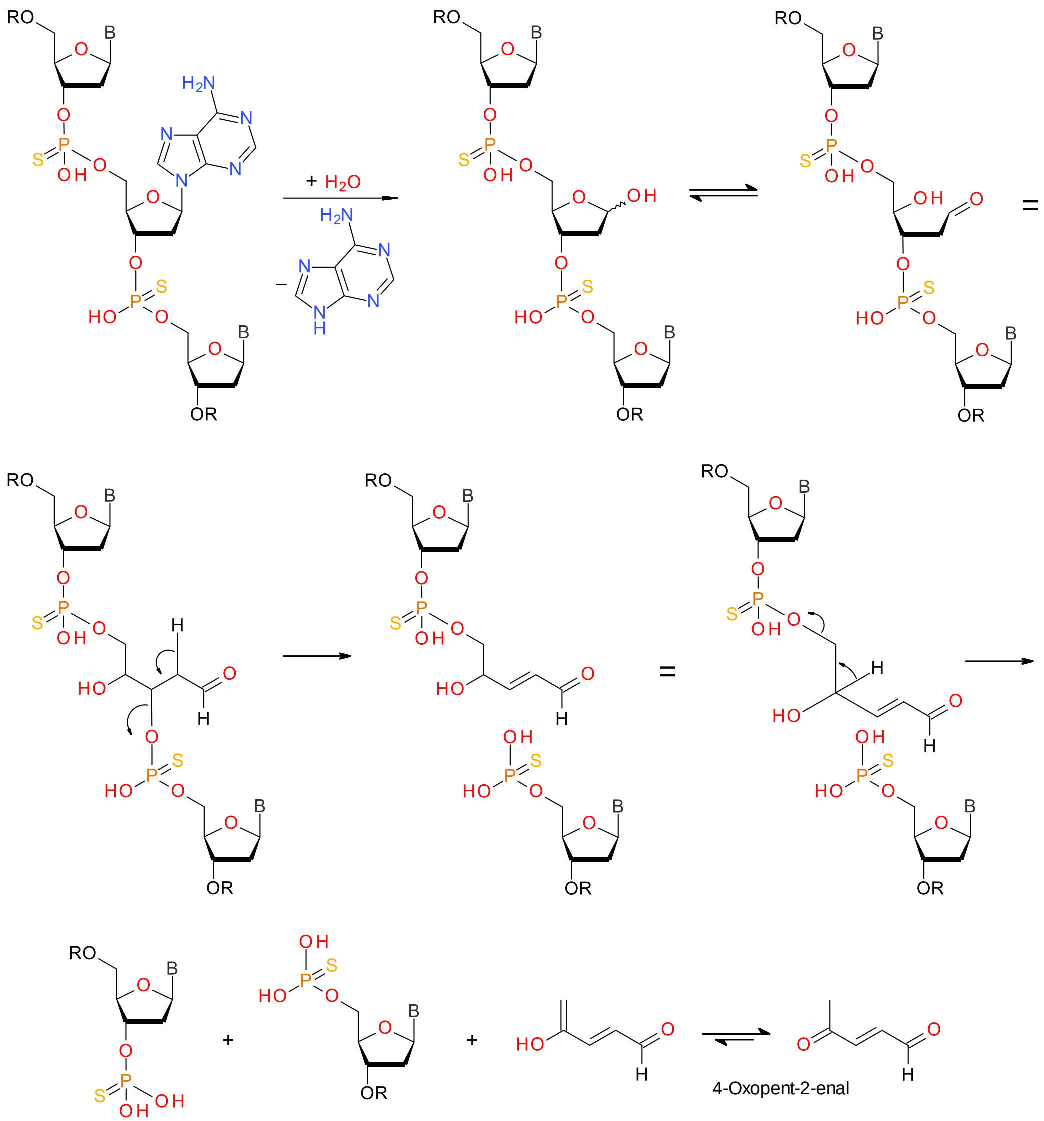
s.(x-end)

- Series of (x-end) impurites resulting form incomplete coupling (failure sequences)

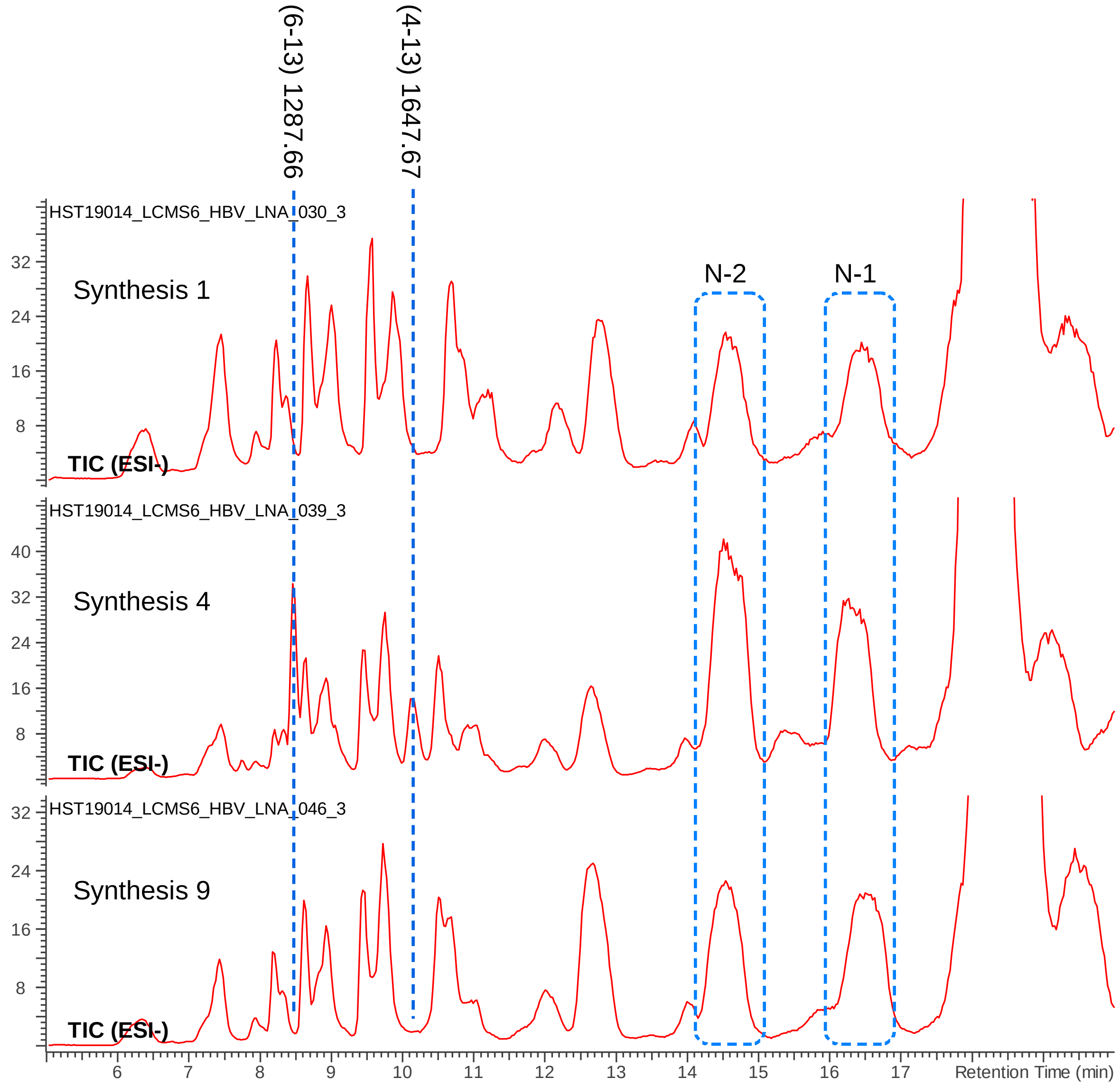
- s.(x-end) TPT/TPO resulting from depurination followed by strand cleavage

- (1-x).s TPT/TPO resulting from depurination followed by strand cleavage

Comparison of the impurity profile of in-process samples



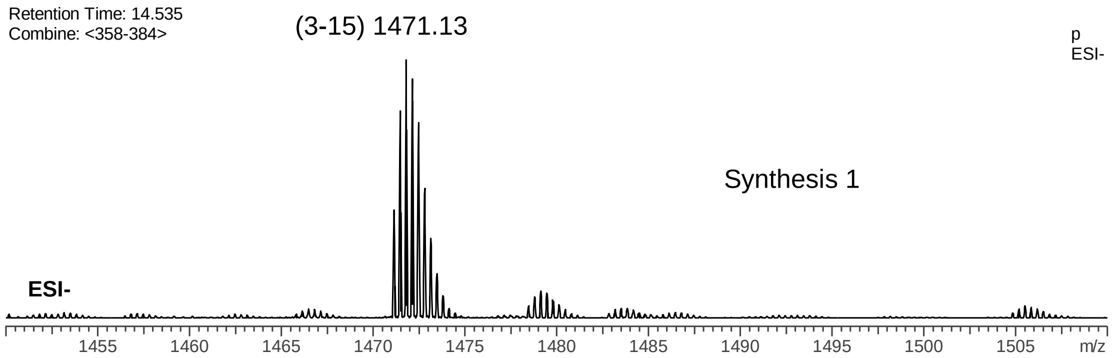
Comparison of the impurity profile of in-process samples

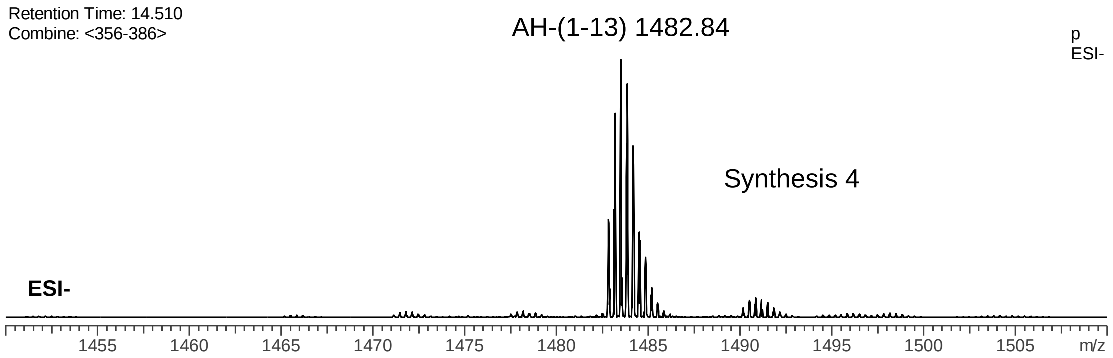


- Synthesis batch #4 gave poor yield
- The corresponding crude sample shows additional peaks
- All N-1 coeluting, could be different sturctures

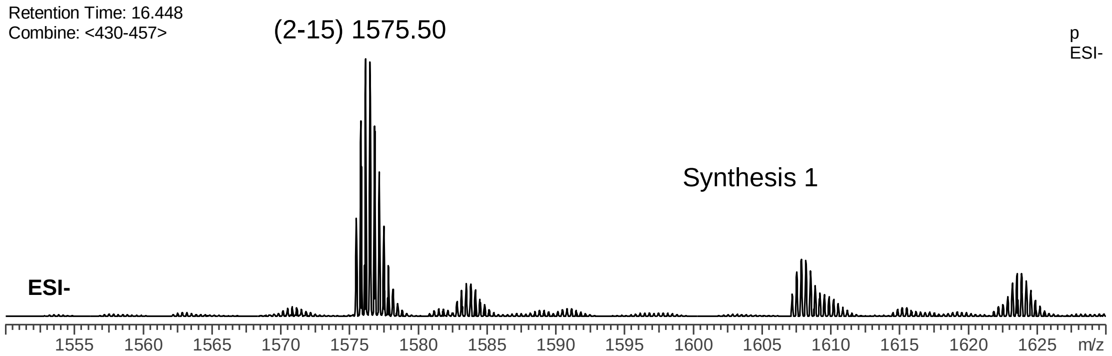
Comparison of the impurity profile of in-process samples

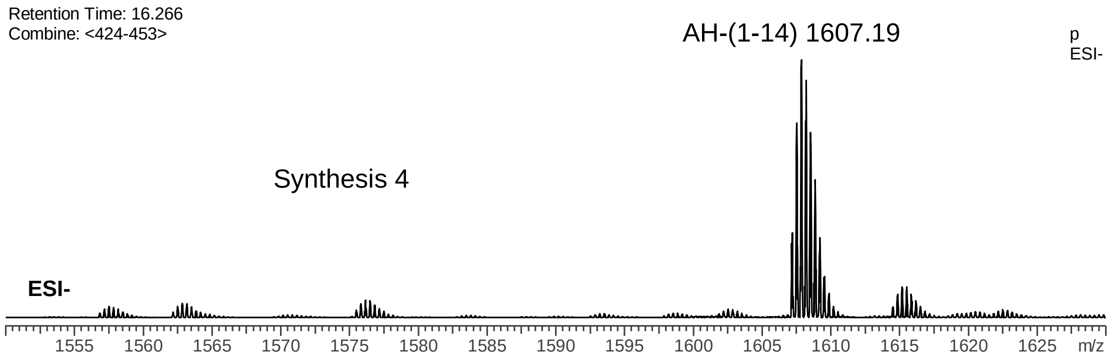
N-2





N-1





Comparison of the impurity profile of in-process samples

N:	Lo.dCo.dAo.**lGs**.lE.**lGs**.dTs.dAs.dAs.dAs.dGs.dAs.dGs.dAs.**lGs.lG**
(3-15):	**lGs**.lE.**lGs**.dTs.dAs.dAs.dAs.dGs.dAs.dGs.dAs.**lGs.lG**
(2-15):	dAo.**lGs**.lE.**lGs**.dTs.dAs.dAs.dAs.dGs.dAs.dGs.dAs.**lGs.lG**
AH-(1-13):	Lo.dCo.dAo.**lGs**.lE.**lGs**.dTs.dAs.dAs.dAs.dGs.dAs.dGs.dA
AH-(1-14):	Lo.dCo.dAo.**lGs**.lE.**lGs**.dTs.dAs.dAs.dAs.dGs.dAs.dGs.dAs.**lG**
(6-13):	dTs.dAs.dAs.dAs.dGs.dAs.dGs.dA
(4-13):	lE.**lGs**.dTs.dAs.dAs.dAs.dGs.dAs.dGs.dA

Occured in synthesis 4

- Concluison:
  - Something went wrong on the LNA-G couplings

Doing now what patients need next