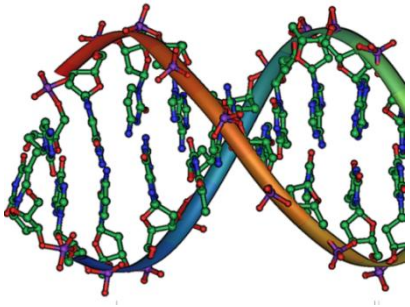
Bioanalytical LC-MS/MS of therapeutic oligonucleotides



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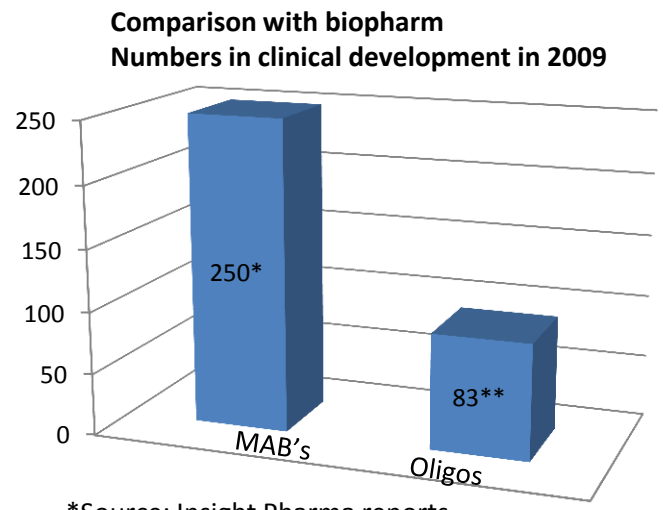
Oligonucleotide therapeutics

- RNA chains 15-25 nucleotide units
- cytosine (C) guanine (G), adenine (A) uracil (U)
- interferes with processing of genetic material
  - inhibits/decreases expression of therapeutically relevant protein
  - blocks expression of virus mRNA
- can treat diseases “undruggable” using small molecules or MABs
  - diseases with a genetic background e.g. DMD, cystic fibrosis, specific cancers and rare diseases
  - common diseases e.g. Hep-C, atherosclerosis, lupus, psoriasis
- Two types
  - single stranded: *e.g.* antisense RNA
  - double stranded: *e.g.* short interfering RNA

Oligonucleotide therapeutics

- Currently two drugs on the market
  - Vitravene® for cytomegalovirus infection *(herpes)*
  - Macugen® for wet macular degeneration *(loss of vision in the center of the visual field)*
- Many in development
  - >250 therapeutic programs
  - >100 in the clinic (2011)
  - >5 in phase III



*Source: Insight Pharma reports

**Source: Agilent Nucleic Acid Solutions

Bioanalysis of oligonucleotides

- Method-of-choice: ELISA
  - based on hybridization of a probe or a capture and/or detection strand complementary to asRNA or siRNA
  - unsurpassed sensitivity (25 pg/ml)
- Drawbacks
  - cannot distinguish full-length oligonucleotides from truncated shortmer metabolites
  - overestimation of parent oligonucleotide
  - Cannot determine intact siRNA

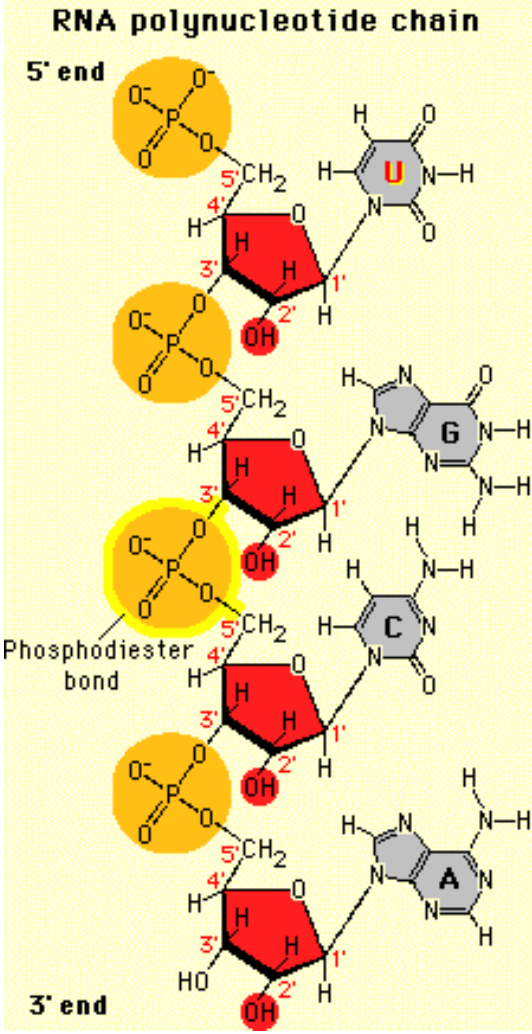
Bioanalysis of oligonucleotides

- LC-MS
  - unsurpassed selectivity : accurate levels
  - indentify and quantify metabolites
- Drawbacks
  - “best” reported sensitivity of validated method: 4 ng/ml [Deng et al, J Pharm Biomed Anal. 52(4), 571-579 (2010)]

Bioanalytical LC-MS/MS of therapeutic oligonucleotides

<u>Highly challenging from an analytical perspective:</u>

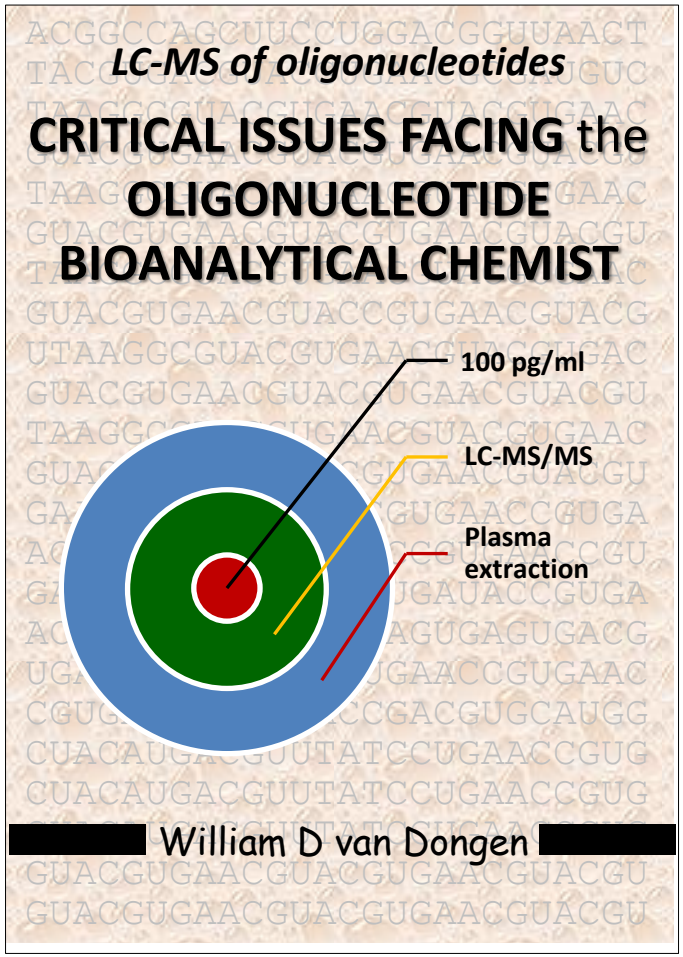
- Acidic proton at each phosphodiester bond
- Highly charged poly-anionic backbone
- Extremely polar
- Phosphorothioate linkage



Bioanalytical LC-MS/MS of oligonucleotides

Critical issues:

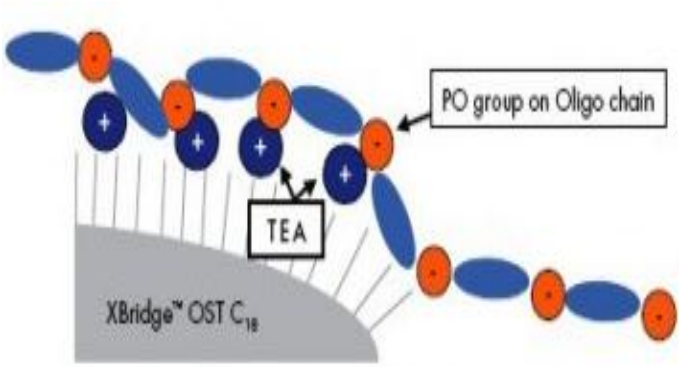
- LC-MS efficiency
  - retention vs. ionisation
- ESI-MS
  - multiple negative charge states
  - H^+-alkali$^+$ exchange at phosphate groups
- MS/MS
  - fragmentation of multiple charged OGNs
- Quantitation
  - internal standard selection
- Sample preparation
  - SPE
  - LLE

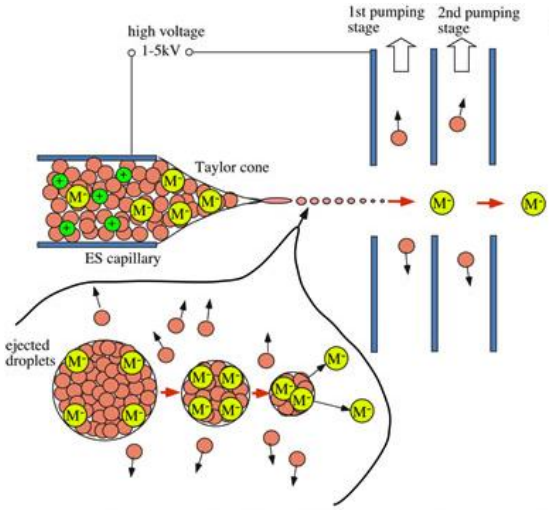


LC-MS efficiency:

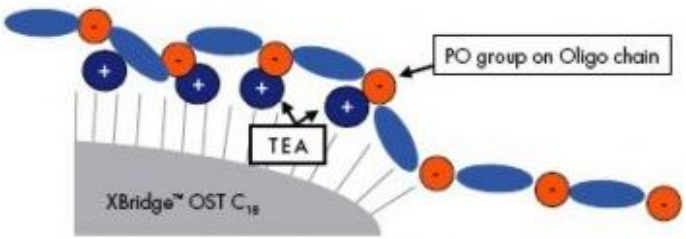
chromatographic retention vs. ionisation efficiency dilemma

LC		**ESI**
low	organic	high
low	pH	high
ionpair	ions	low





IPLC-MS of oligonucleotides

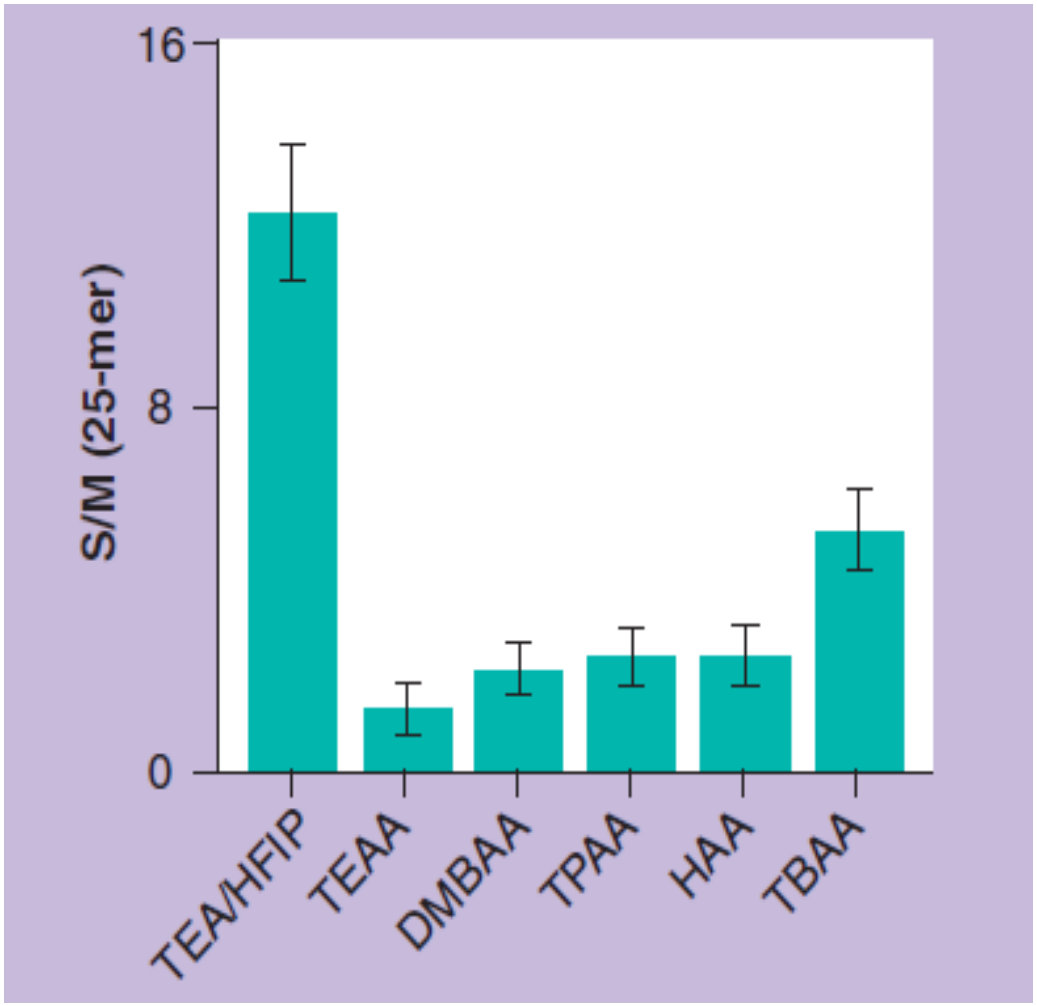


- C18 column
- 10 mM triethylamine (TEA)
- 100 mM hexafluoro-2-propanol (HFIP)
- MeOH/MeCN gradient

IPLC-MS of oligonucleotides

- LC: HFIP increases hydrophobicity ion-pair
- ESI: HFIP dynamic liquid/gas phase pH adjuster
  - pK_a 9, 99% not charged at pH 7, bp 57◦C
  - evaporates during ESI
  - volatile HFIP depletes at droplet surface
  - pH at the surface rises to 10
  - OGN-TEA ion pair dissociation
  - desorption OGN into the gas phase.
- And: HFIP reduces cation exchange

Single-quadrupole S/N ratios (n=3) of 20 pg (dT)25 IPLC-MS

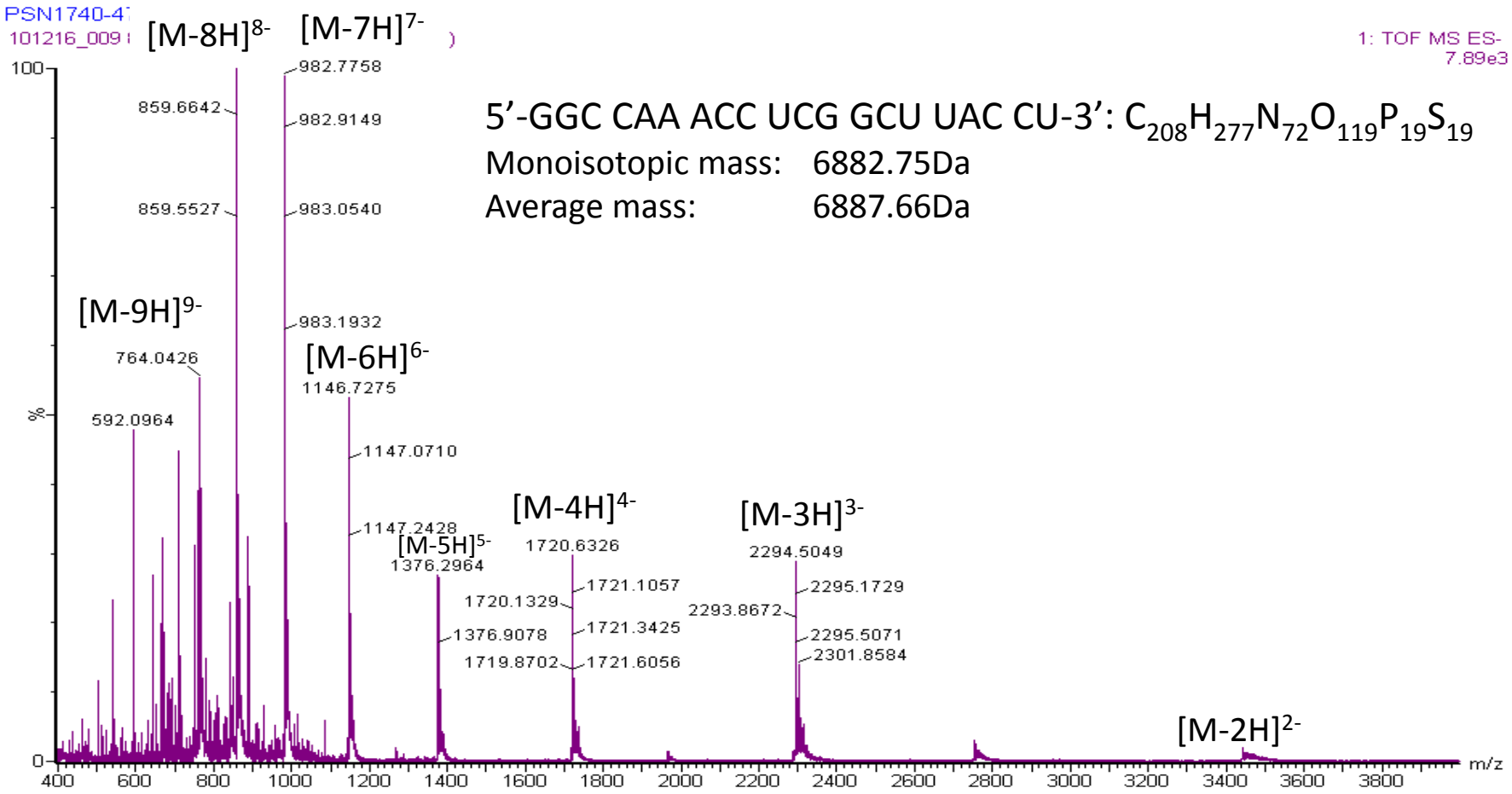


TEA/HFIP:
15mM triethylammonium /400mM hexafluoro-2-propanol
TEAA:
100mM triethylammonium acetate
DMBAA:
100mM butyldimethylammonium ac.
TPAA:
100mM tripropylammonium acetate
TBAA:
100mM tributylammonium acetate
HAA:
100mM hexyl ammonium acetate

McCarthy *et al. 5th Symposium on the Practical Applications of Mass Spectrometry in the Biotechnology and Pharmaceutical Industries.* The Meritage Resort, Napa, CA, USA, 9–11 September 2008

ESI-MS:

formation of multiple negative charge states



5'-GGC CAA ACC UCG GCU UAC CU-3': $C_{208}H_{277}N_{72}O_{119}P_{19}S_{19}$

Monoisotopic mass: 6882.75Da

Average mass: 6887.66Da

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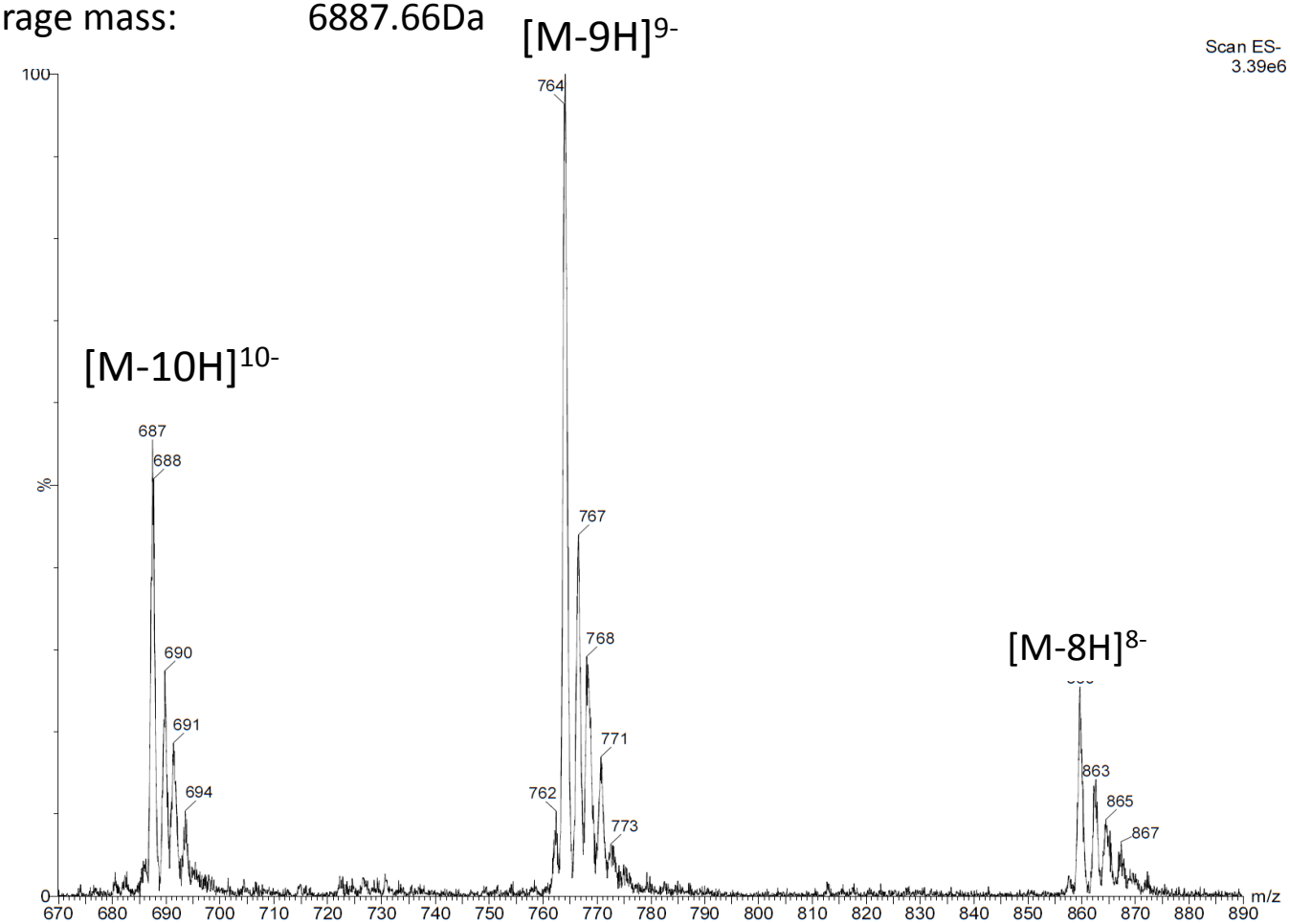
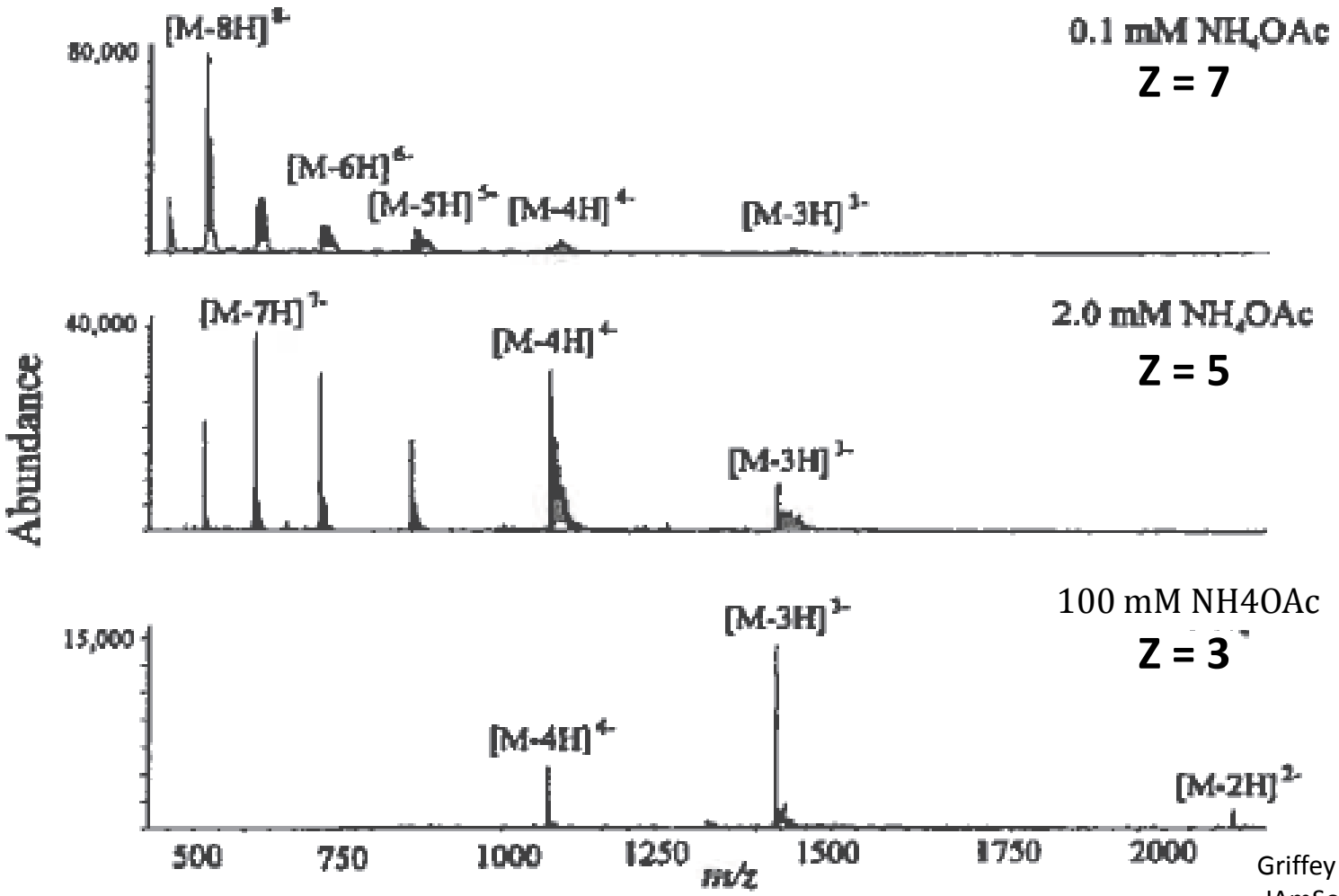
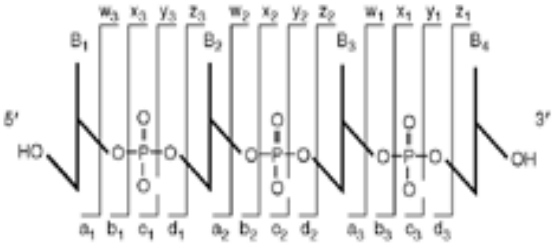


5’-GAGACTGCAAGCG-3’



Griffey *et al*, JAmSoc 8, 155-160 (1997)

Complex MS/MS spectra



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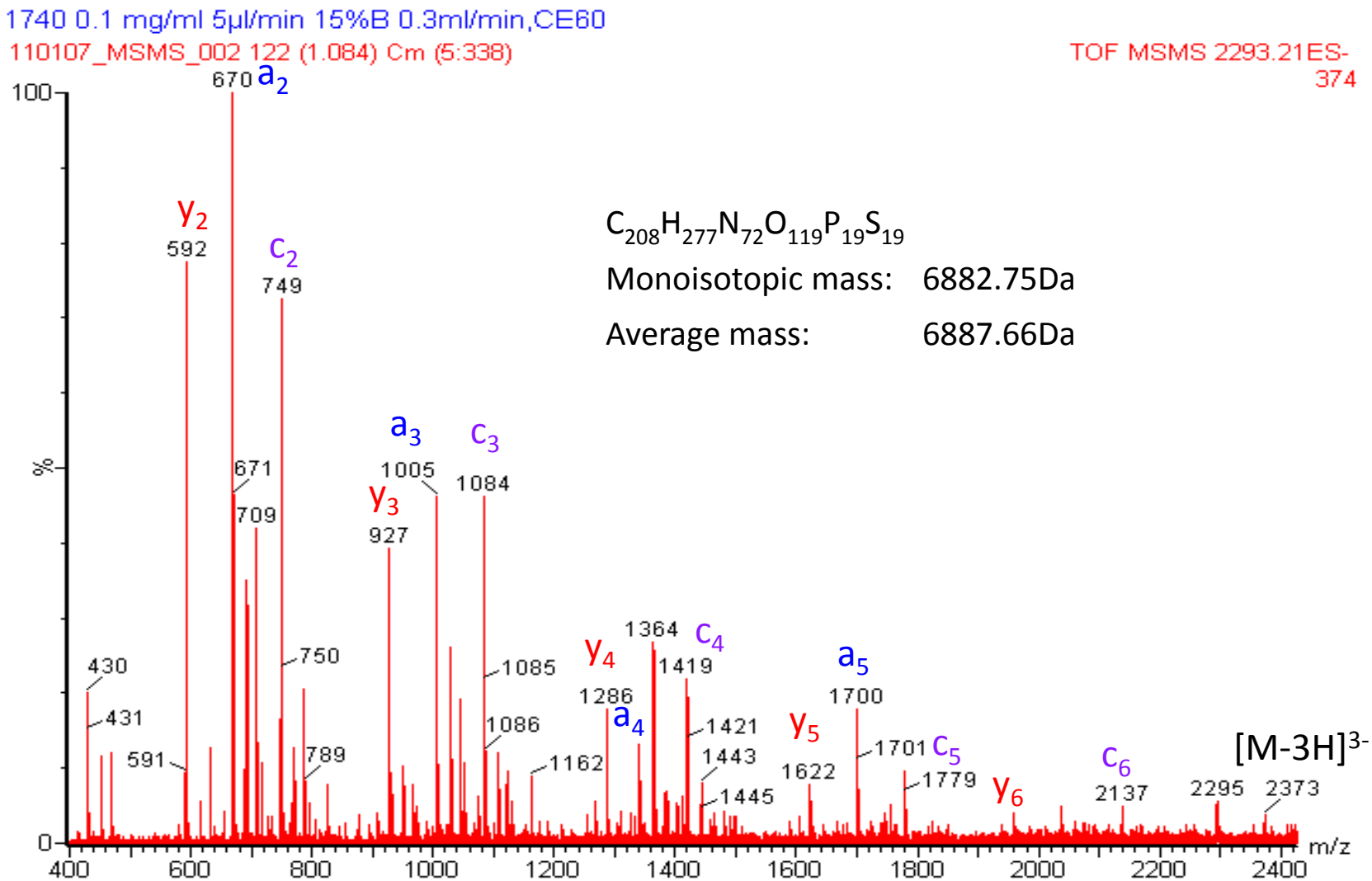
1648,6	1554,8	1471,0	1387,1	1297,3	1207,5	1117,7	1033,8	950,0	866,0	782,1	688,3	594,5	510,7	426,6	342,5	252,7					4
2198,5	2073,4	1961,6	1849,8	1730,1	1610,3	1490,5	1378,8	1267,0	1154,9	1043,2	918,1	793,0	681,2	569,1	457,0	337,3	225,5				3
3298,2	3110,5	2942,9	2775,3	2595,6	2416,0	2236,3	2068,7	1901,0	1732,9	1565,3	1377,6	1190,0	1022,3	854,2	686,1	506,4	338,8				2
6597,4	6222,1	5886,8	5551,5	5192,2	4832,9	4473,6	4138,3	3803,1	3466,8	3131,5	2756,2	2380,9	2045,6	1709,4	1373,1	1013,8	678,5	343,3			1w-ion
1695,1	1601,3	1517,5	1433,6	1343,8	1254,0	1164,2	1080,3	996,5	912,5	828,6	734,8	641,0	557,2	473,1	389,0	299,2					4
2237,8	2112,7	2000,9	1889,2	1769,4	1649,6	1529,9	1418,1	1306,4	1194,3	1082,5	957,4	832,3	720,5	608,5	496,4	376,6	264,8				3
3323,2	3135,5	2967,9	2800,3	2620,6	2441,0	2261,3	2093,7	1926,0	1757,9	1590,3	1402,6	1215,0	1047,3	879,2	711,1	531,4	329,8				2
6579,4	6204,1	5868,8	5533,5	5174,2	4814,9	4455,6	4120,3	3785,1	3448,8	3113,5	2738,2	2362,9	2027,6	1691,4	1355,1	995,8	660,5	325,3			1x-ion
1627,1	1533,3	1449,5	1365,6	1275,8	1186,0	1096,2	1012,3	928,5	844,5	760,6	666,8	573,0	489,2	405,1	321,0	231,2					4
2169,8	2044,7	1932,9	1821,2	1701,4	1581,6	1461,9	1350,1	1238,4	1126,3	1014,5	889,4	764,3	652,5	540,5	428,4	308,6	196,8				3
3255,2	3067,5	2899,9	2732,3	2552,6	2373,0	2193,3	2025,7	1858,0	1689,9	1522,3	1334,6	1147,0	979,3	811,2	643,1	463,4	295,8				2
6511,4	6136,1	5800,8	5465,5	5106,2	4746,9	4387,6	4052,3	3717,1	3380,8	3045,5	2670,2	2294,9	**1959,6**	**1623,4**	**1287,1**	**927,8**	**592,5**	257,3			1y-ion
1622,6	1528,8	1444,9	1361,1	1271,3	1181,5	1091,6	1007,8	924,0	839,9	756,1	662,3	568,5	484,7	400,6	316,5	226,7					4
2163,8	2038,7	1926,9	1815,2	1695,4	1575,6	1455,9	1344,1	1232,4	1120,3	1008,5	883,4	758,3	646,5	534,5	422,4	302,6	190,8				3
3246,2	3058,5	2890,9	2723,3	2543,6	2364,0	2184,3	2016,7	1849,0	1680,9	1513,3	1325,6	1138,0	970,3	802,2	634,1	454,4	286,8				2
6493,4	6118,1	5782,8	5447,5	5088,2	4728,9	4369,6	4034,3	3699,1	3362,8	3027,5	2652,2	2276,9	1941,6	**1605,4**	**1269,1**	**909,8**	574,5	239,3			1z-ion

		5'	G	G	C	C	A	A	A	C	C	U	C	G	G	C	U	U	A	C	C	U	3'	mol mass
	res-mass	17,0	375,3	375,3	335,3	335,3	359,3	359,3	359,3	335,3	335,3	336,3	335,3	375,3	375,3	335,3	336,3	336,3	359,3	335,3	335,3	336,3	-95,0	6887,7
	Base		151,1	151,1	111,0	111,0	135,1	135,1	135,1	111,0	111,0	112,0	111,0	151,1	151,1	111,0	112,0	112,0	135,1	111,0	111,0	112,0		
a-ions	1		295,3	**670,6**	**1005,9**	**1341,2**	**1700,5**	2059,8	2419,1	2851,4	3089,6	3425,9	3761,2	4136,5	4511,8	4847,1	5183,3	5519,6	5878,9	6214,2	6549,4	6885,7		
	2			334,8	502,4	670,1	849,7	1029,4	1209,0	1425,2	1544,3	1712,4	1880,1	2067,7	2255,4	2423,0	2591,2	2759,3	2938,9	3106,6	3274,2	3442,3		
	3				334,6	446,4	566,2	685,9	805,7	949,8	1029,2	1141,3	1253,1	1378,2	1503,3	1615,0	1727,1	1839,2	1959,0	2070,7	2182,5	2294,6		
	4					334,5	424,4	514,2	604,0	712,1	771,7	855,7	939,5	1033,4	1127,2	1211,0	1295,1	1379,1	1469,0	1552,8	1636,6	1720,7		
a-B ions	1		144,3	519,6	**894,8**	**1230,1**	1565,4	1924,7	2284,0	2740,3	2978,6	3313,9	3650,1	3985,4	4360,7	4736,0	5071,3	5407,6	5743,8	6103,1	6438,4	6773,7		
	2			259,3	446,9	614,6	782,2	961,9	1141,5	1369,7	1488,8	1656,4	1824,6	1992,2	2179,9	2367,5	2535,1	2703,3	2871,4	3051,1	3218,7	3386,3		
	3				297,6	409,4	521,1	640,9	760,7	912,8	992,2	1104,0	1216,0	1327,8	1452,9	1578,0	1689,8	1801,9	1913,9	2033,7	2145,5	2257,2		
	4					306,8	390,6	480,4	570,3	684,3	743,9	827,7	911,8	995,6	1089,4	1183,3	1267,1	1351,1	1435,2	1525,0	1608,8	1692,7		
C ions	1		374,3	**749,6**	**1084,9**	**1420,2**	**1779,5**	**2138,8**	**2498,1**	**2833,3**	**3168,6**	**3504,9**	**3840,2**	4215,5	4590,8	4926,0	5262,3	5598,6	5957,9	6293,1	6628,4	6964,7		
	2			374,3	541,9	709,6	889,2	1068,9	1248,5	1416,2	1583,8	1751,9	1919,6	2107,2	2294,9	2462,5	2630,6	2798,8	2978,4	3146,1	3313,7	3481,8		
	3				361,0	472,7	592,5	712,2	832,0	943,8	1055,5	1167,6	1279,4	1404,5	1529,6	1641,3	1753,4	1865,5	1985,3	2097,0	2208,8	2320,9		
	4					354,3	444,1	533,9	623,8	707,6	791,4	875,5	959,3	1053,1	1146,9	1230,8	1314,8	1398,9	1488,7	1572,5	1656,4	1740,4		
b-ions	1		313,3	688,6	**1023,9**	1359,2	1718,5	2077,8	2437,1	2869,4	3107,6	3443,9	3779,2	4154,5	4529,8	4865,1	5201,3	5537,6	5896,9	6232,2	6567,4	6903,7		
	2			343,8	511,4	679,1	858,7	1038,4	1218,0	1434,2	1553,3	1721,4	1889,1	2076,7	2264,4	2432,0	2600,2	2768,3	2947,9	3115,6	3283,2	3451,3		
	3				340,6	452,4	572,2	691,9	811,7	955,8	1035,2	1147,3	1259,1	1384,2	1509,3	1621,0	1733,1	1845,2	1965,0	2076,7	2188,5	2300,6		
	4					339,0	428,9	518,7	608,5	716,6	776,2	860,2	944,0	1037,9	1131,7	1215,5	1299,6	1383,6	1473,5	1557,3	1641,1	1725,2		

5’-GGCCAAACCUCGCUUACCU-3’

MS/MS of $[M-3H]^{3-}$



$C_{208}H_{277}N_{72}O_{119}P_{19}S_{19}$

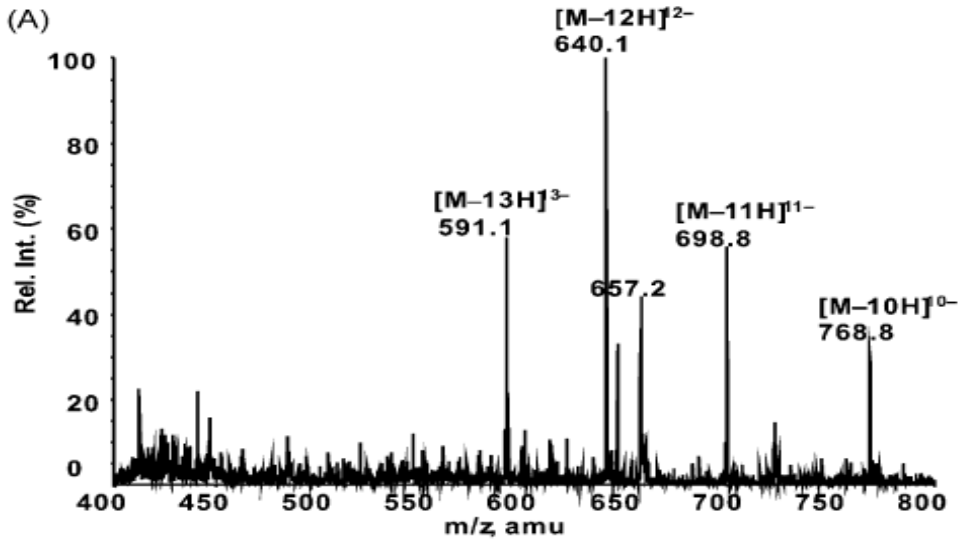
Monoisotopic mass: 6882.75Da

Average mass: 6887.66Da

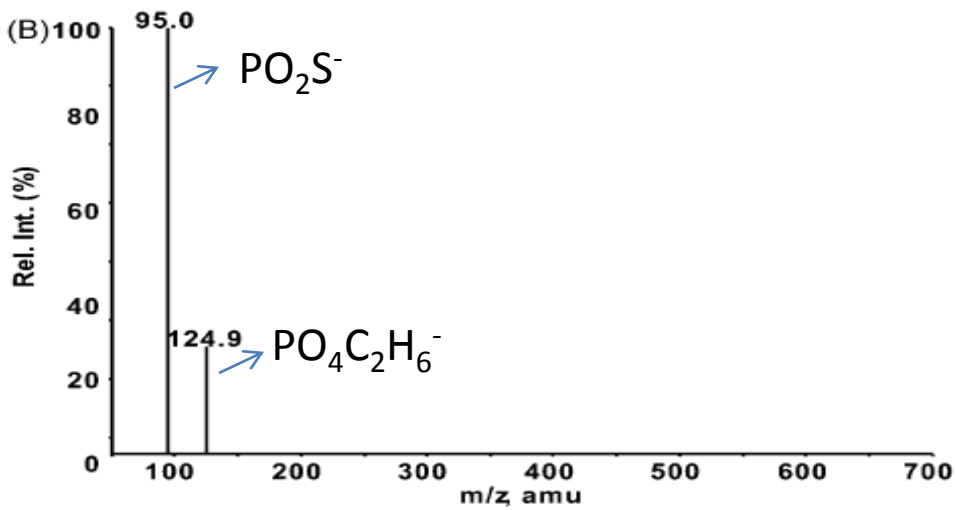
5’-TCTCCCAGCGTGCGCCAT-3’

MS/MS of [M-13H]$^{13-}$

Full scan m/z 400-800



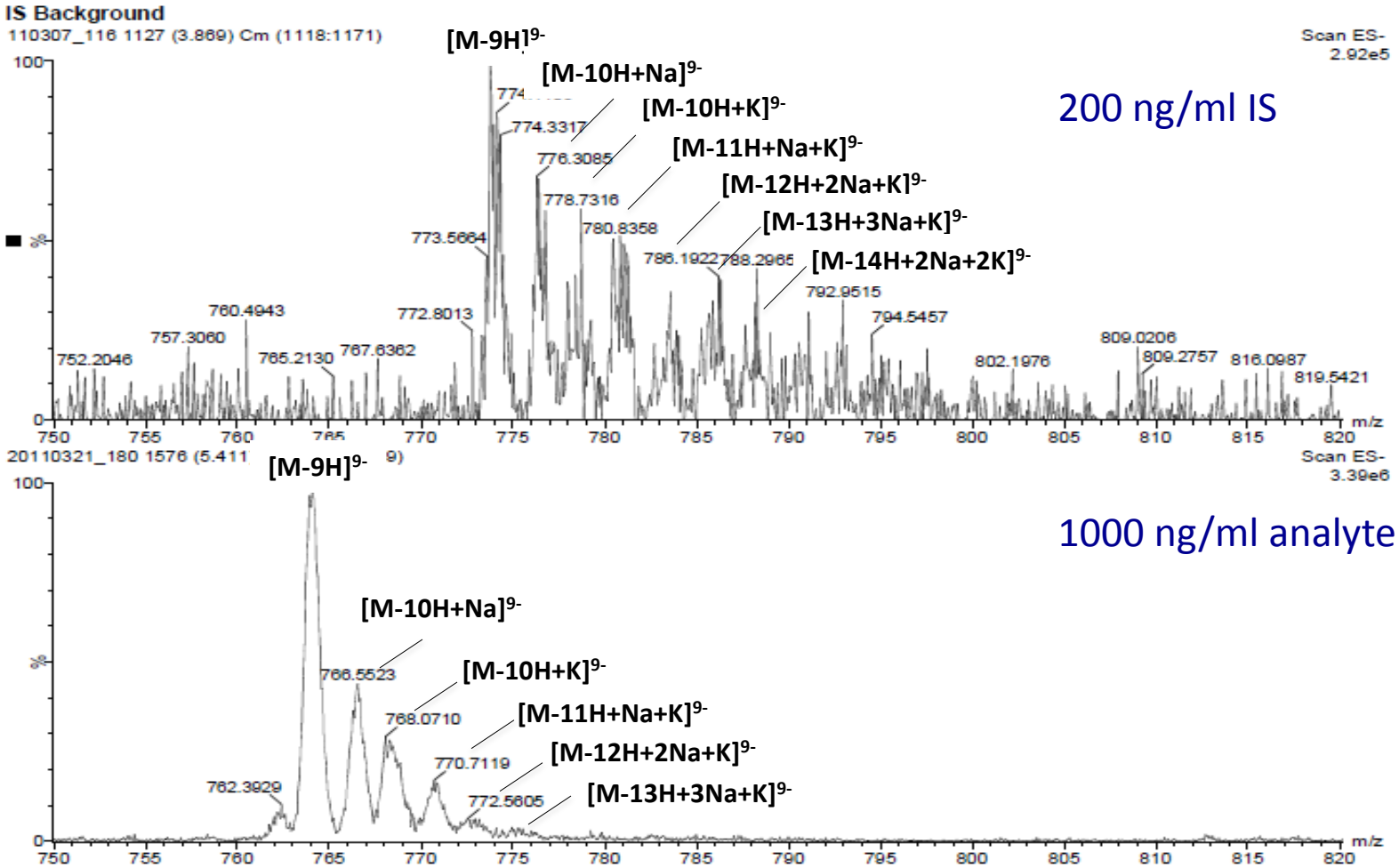
product ion scan of m/z 591 [M–13H]$^{13-}$



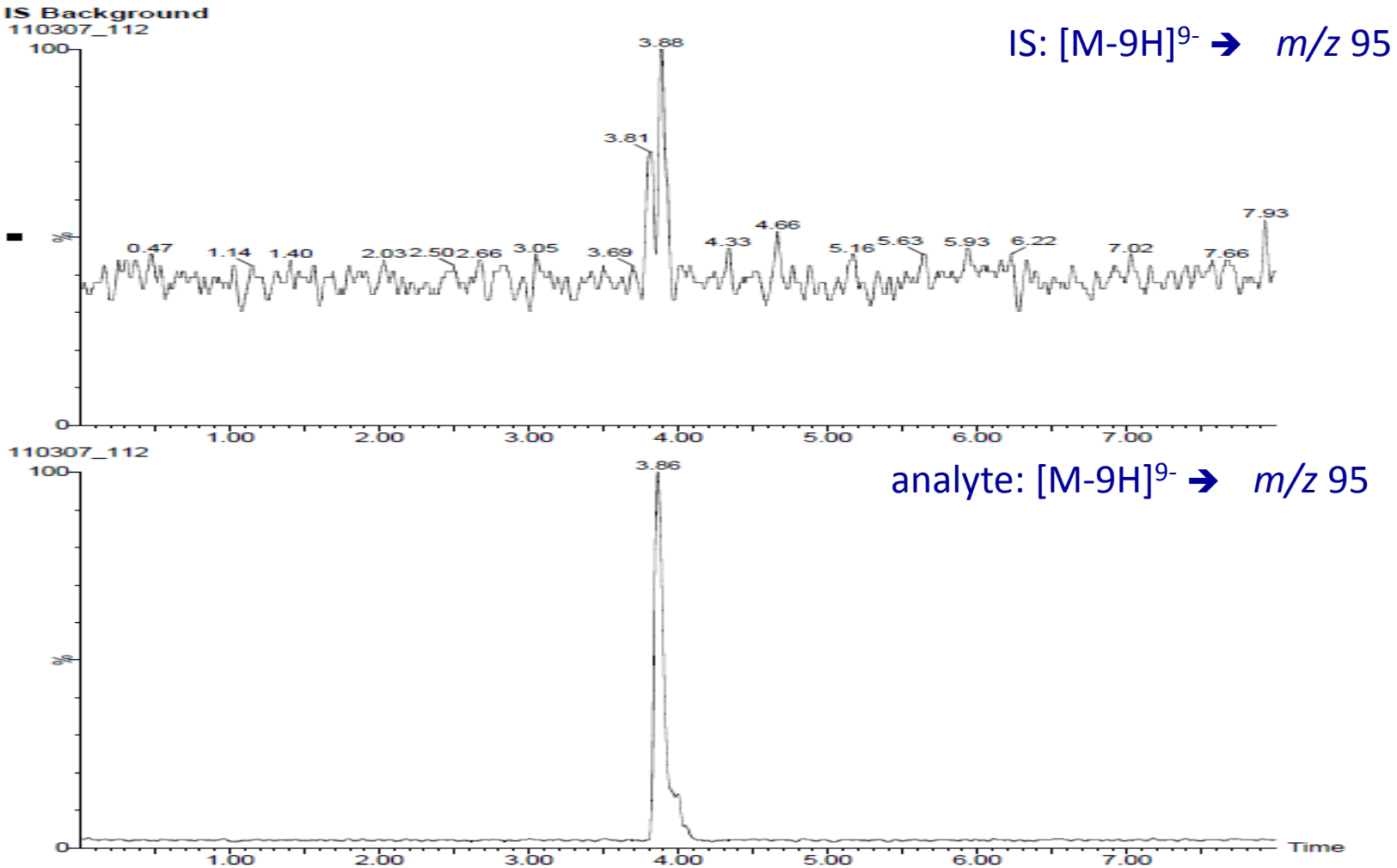
Deng et al, J Pharm Biomed Anal. 52(4), 571-579 (2010)

ESI-MS

H^+-alkali$^+$ exchange at phosphate groups



Quantitation: internal standard selection



Internal standard affairs: case 1

Drug substance:

rafAON $G_STGCTCCATTGATG_SC$ mol mass: 4590

IS $G_SUGCUCCAUUGAUG_SC$ mol mass: 4521

Chemical modifications:

lower case S = PS linkage

LC:

no separation of rafAON and IS

SRM:

rafAON, $[M-3H]^{3-}$: 1529 → 322 + 1529 → 746

IS , $[M-3H]^{3-}$: 1506 → 289

LOQ: 50 ng/ml

Internal standard affairs: case 2

Drug substance:		**mol mass:**
PF-ODN	TCGTCGTTTTGTCGTTTTGTCGTT	7697
IS:	TTTTTTTTTTTTTTTTTTTTTT	6327

LC:

no separation of PF-ODN and IS

SRM:

PF-ODN, $[M-xH]^{x-}$ 698.8, 640.1, 591.1 (n=11-13) → 95

IS , $[M-10H]^{10-}$: 631.7 (n=10) → 125

LOQ: 4 ng/ml

Deng et al, J Pharm Biomed Anal. 52(4), 571-579 (2010)

Sample preparation of plasma samples

drug	sample prep	recovery	suppression
	SPE		
G3139	OASIS HLB	40-50%	na
rafAON	OASIS HLB	22.8 ± 6.5%	48.2 ± 3.5%
rafAON	Varian C18	80%	50%
Compound X	OASIS WAX	75%	na
Oligo1	Clarity OTX	>80%	na
	LLE		
Compounds A/B/C	phenol/ chloroform/ isoamyl alcohol	80%	na
	LLE & SPE		
PF-ODN	chlorof/phenol & Oasis HLB	70-80%	0-6%

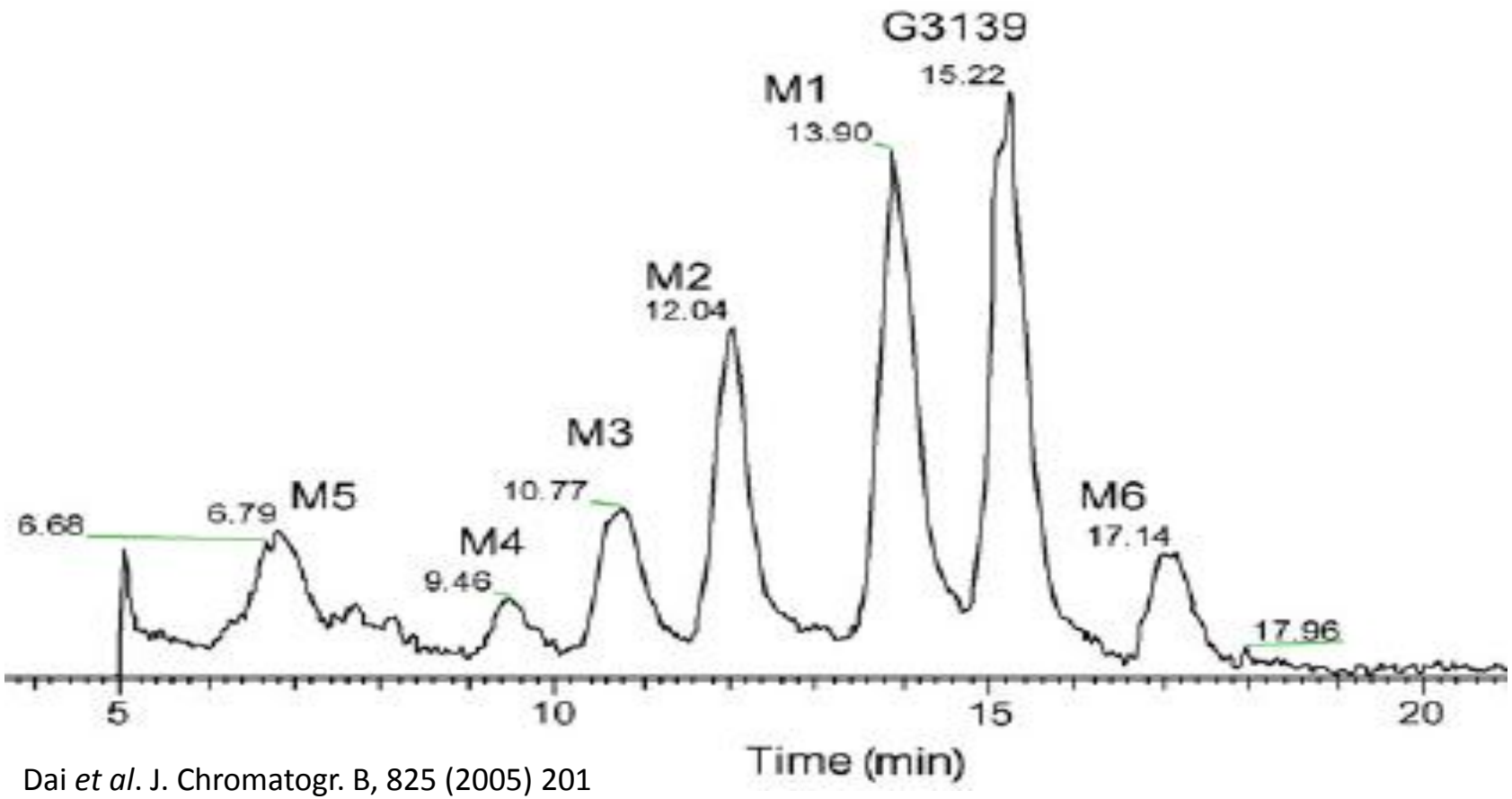
WD van Dongen and WMA Niessen, *Bioanalysis* (2011) 3(5), 541-564

Bioanalytical LC-MS methods of asRNA

drug	sample prep	range	validation statistics
ISIS 1083 21-mer	SPE Phenyl	5-500 ng/ml	89-107% RSD 2-15%
rafAON 15-mer	monkey (OASIS HLB) mouse (OASIS C18)	50-10,000 ng/ml 25-5,000 ng/ml	94–102% RSD 6–14% 95–101% RSD 3–11%
PF-ODN 24-mer 5'-TCGTCGTTTTGTCGTTTTGTCGTT-3'	LLE, chloroform/phenol & Oasis HLB	4-2.000 ng/ml	**PF-ODN**: 97-101% RSD 2-12% **(n-1)5'/(n-1)3'**: 102-106% RSD 1-12% **(n-2)5'**: 99 - 94% RSD 1-12 % **(n-3)5'**: 90– 100 % RSD 6-7%

WD van Dongen and WMA Niessen, *Bioanalysis* (2011) 3(5), 541-564

Metabolites of antisense G3139 in human plasma obtained from *in vivo* study

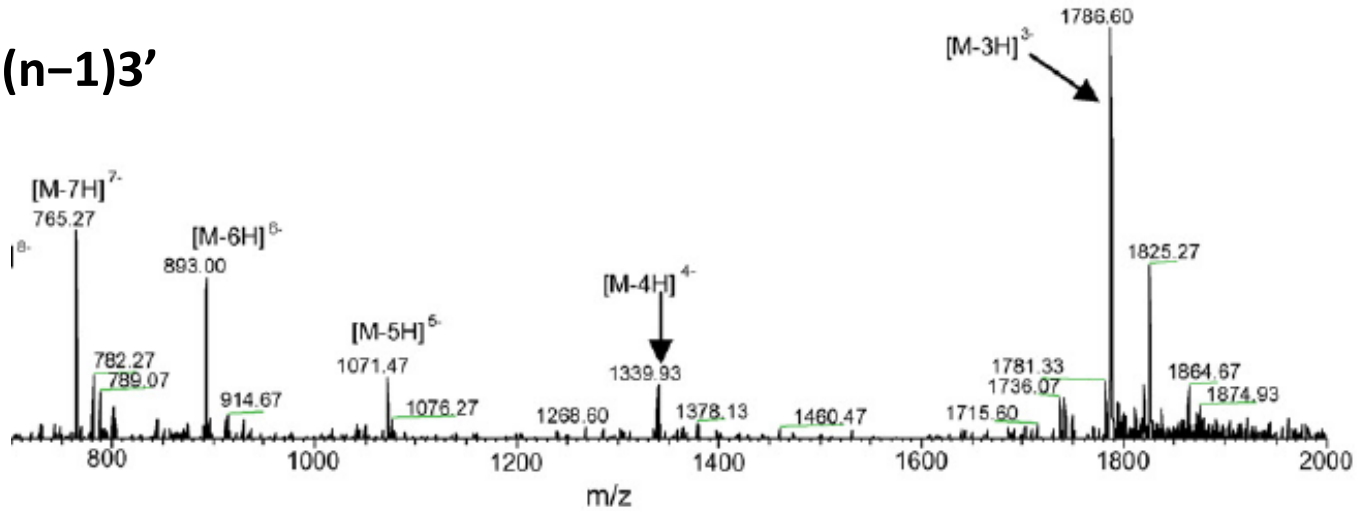


Dai *et al*. J. Chromatogr. B, 825 (2005) 201

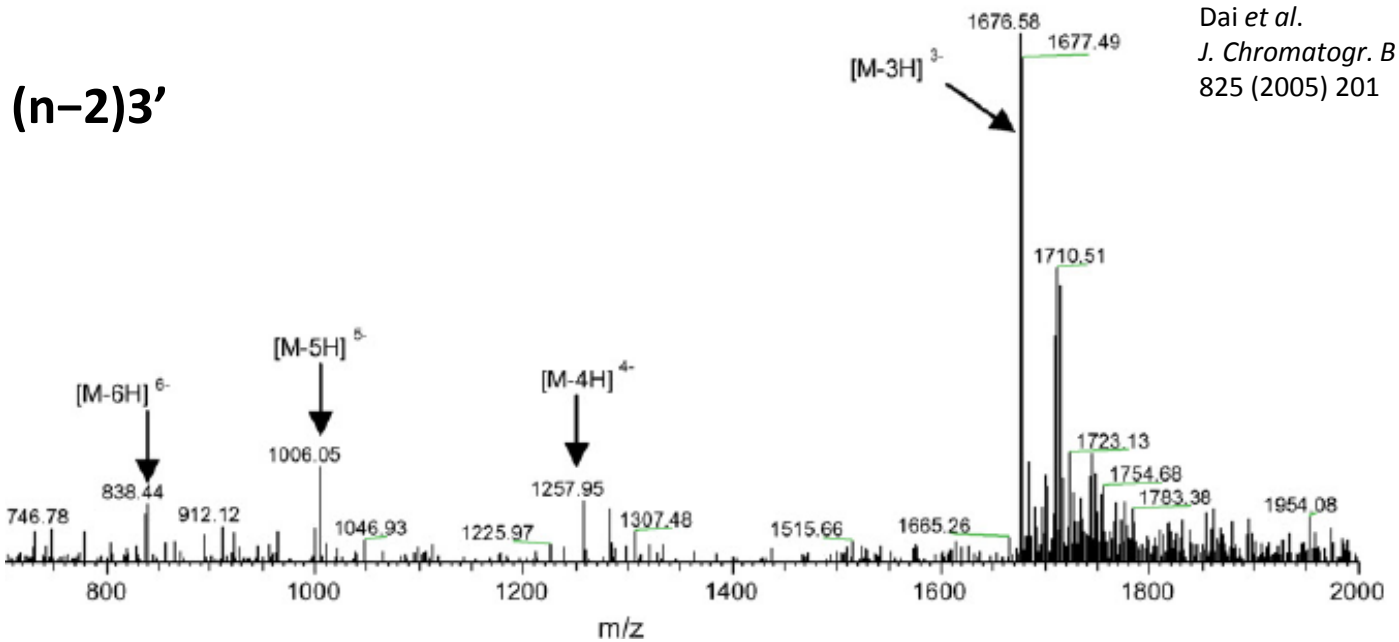
Human metabolites of G3139

G3139 5'TCTCCCAGCGTGCGCCAT'3

(n−1)3'



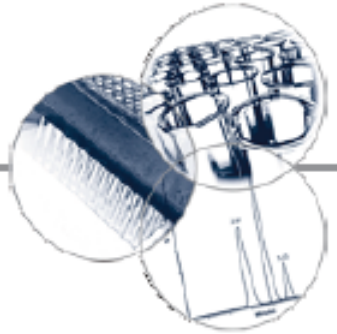
(n−2)3'



Dai *et al*.
J. Chromatogr. B
825 (2005) 201

Future perspective: asRNA

- Current 4 ng/ml, must and will be improved
  - UPLC
  - sensitive triple quadrupole MS (Xevo TQS, API 5500, Agilent 6490)
  - (nano-)UPLC and chip technology
  - make LLQ's in the range of low pg/ml potentially possible



Bioanalytical LC–MS of therapeutic oligonucleotides

Therapeutic oligonucleotides (OGNTs) are important biopharmaceutical drugs for the future, due to their ability to selectively reduce or knockout the expression of target genes. For the development of OGNTs, reliable and relatively high-throughput bioanalytical methods are required to perform the quantitative bioanalysis of OGNTs and their metabolites in biological fluids (e.g., plasma, urine and tissue). Although immunoaffinity methods, especially ELISA, are currently widely applied for this purpose, the potential of LC–MS in OGNT analysis is under investigation. Owing to its inherent ability to monitor the individual target OGNTs as well as their metabolites, LC–MS is now evolving into the method-of-choice for the bioanalysis of OGNTs. In this paper, the state-of-the-art of bioanalytical LC–MS of OGNTs and their metabolites in biological fluids is critically reviewed and its advantages and limitations highlighted. Finally, the future perspective of bioanalytical LC–MS, that is, lower detection levels and potential generic LC–MS methodology, is discussed.

At present, short-chain oligomers are applied and under investigation as **oligonucleotide therapeutics** (OGNTs), typically between 15 and 50 nucleotide units long. These OGNTs are built to interfere with the processing of genetic information by acting on DNA or RNA [1]. Unlike many other drugs, which target the functioning of proteins by interfering with their receptor site, OGNTs target the gene directly or at the mRNA-expression stage and thereby interfere with the production of

Another type of OGNTs is based on a naturally occurring gene silencing mechanism called RNA interference (RNAi), where short dsRNAs knock down gene expression in cells [2–5]. The synthetic 19- to 25-base pair (bp) dsRNAs used as OGNTs of this type are called **short interfering RNA** (siRNAs). The RNAi process is a multistep process: the double-stranded siRNAs are incorporated into the RNA-induced silencing complex (RISC). The central defining component of the RISC is formed by an Argonaute

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WD van Dongen and WMA Niessen, *Bioanalysis* (2011) 3(5), 541-564

Acknowledgements

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Prosensa therapeutics