Electronic Supplementary Information

N-Branched acyclic nucleoside phosphonates as monomers for the synthesis of modified oligonucleotides

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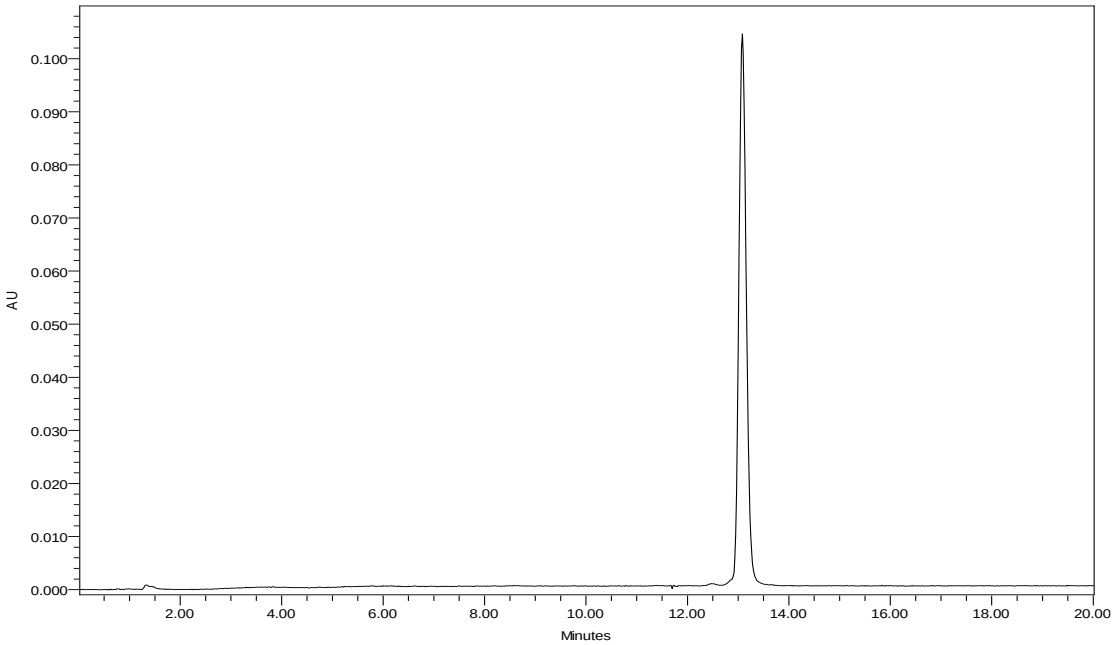
IE HPLC purity of the prepared modified nonamers

dON-2



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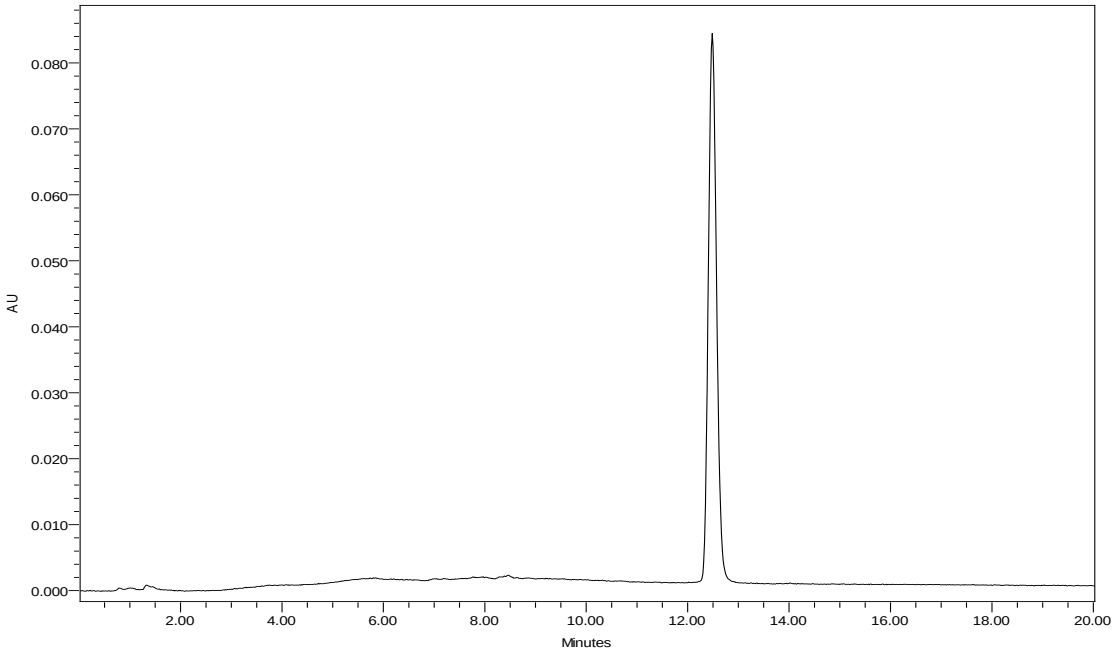
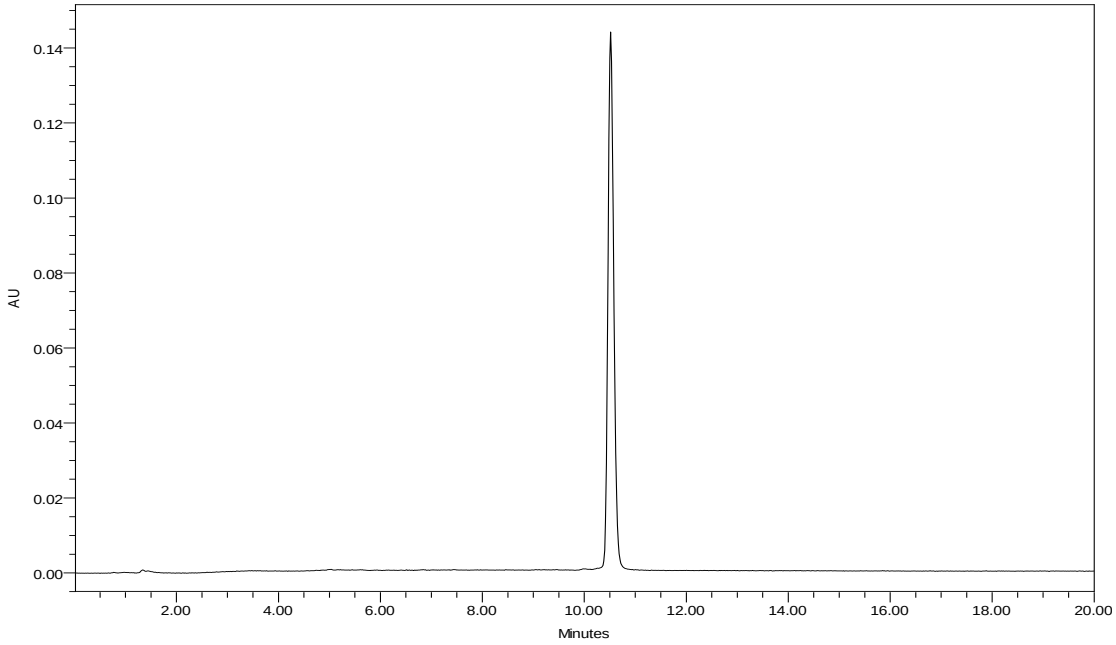


dON-5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0.12
0.10
0.08
0.06
0.04
0.02
0.00
2.00
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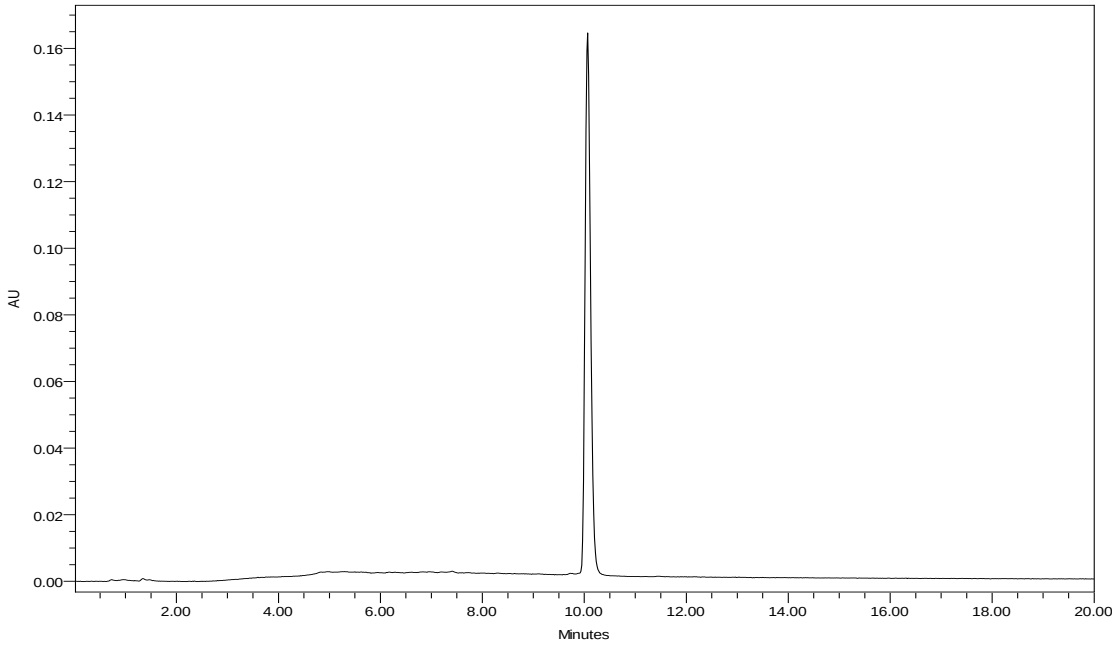
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Minutes

dON-6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16.00
18.00
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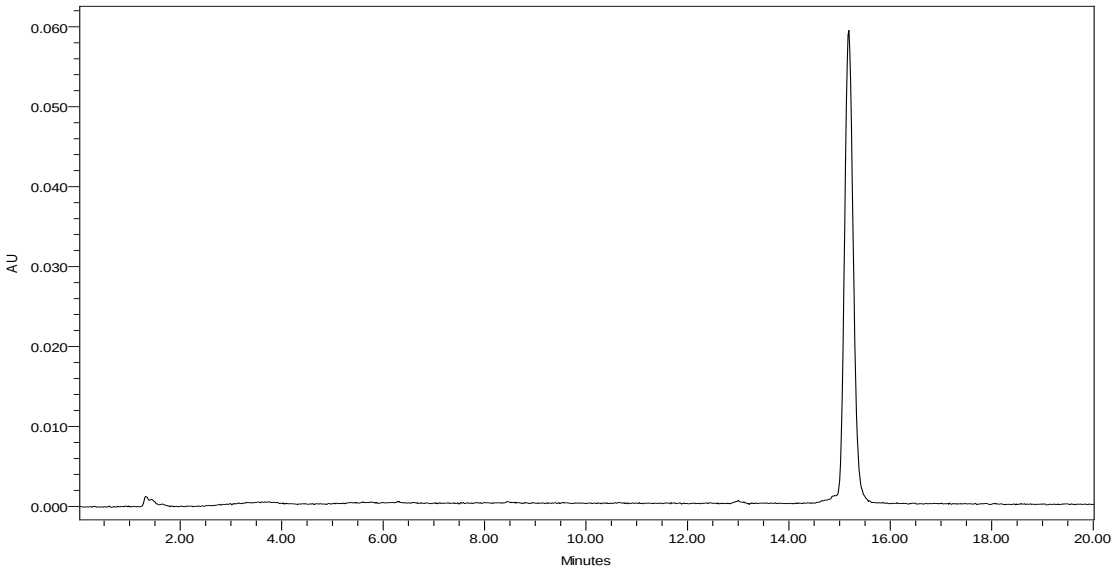
Minutes

rON-8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16.00
18.00
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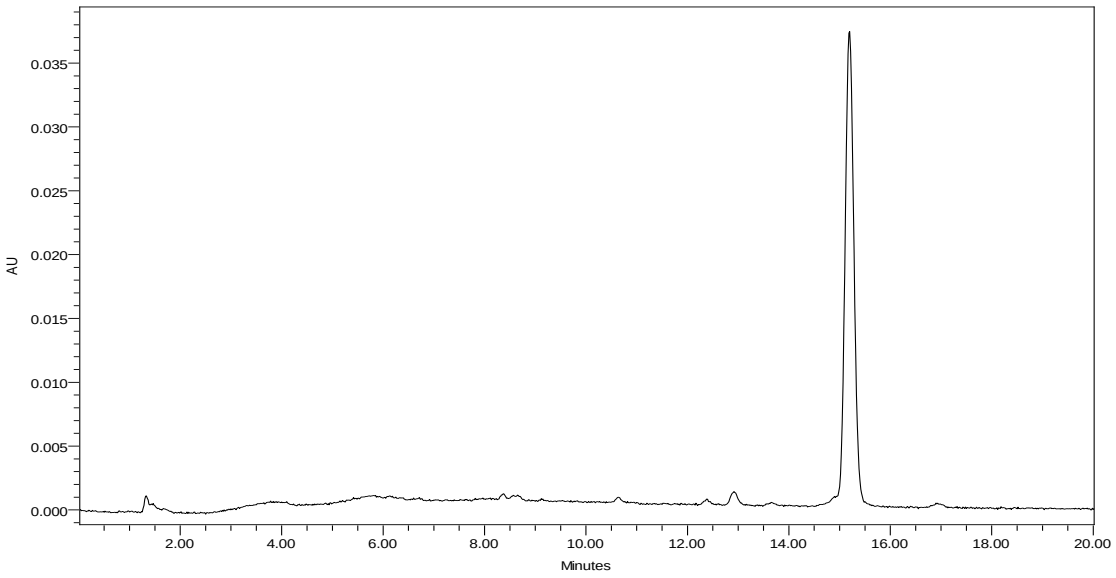
Minutes

rON-9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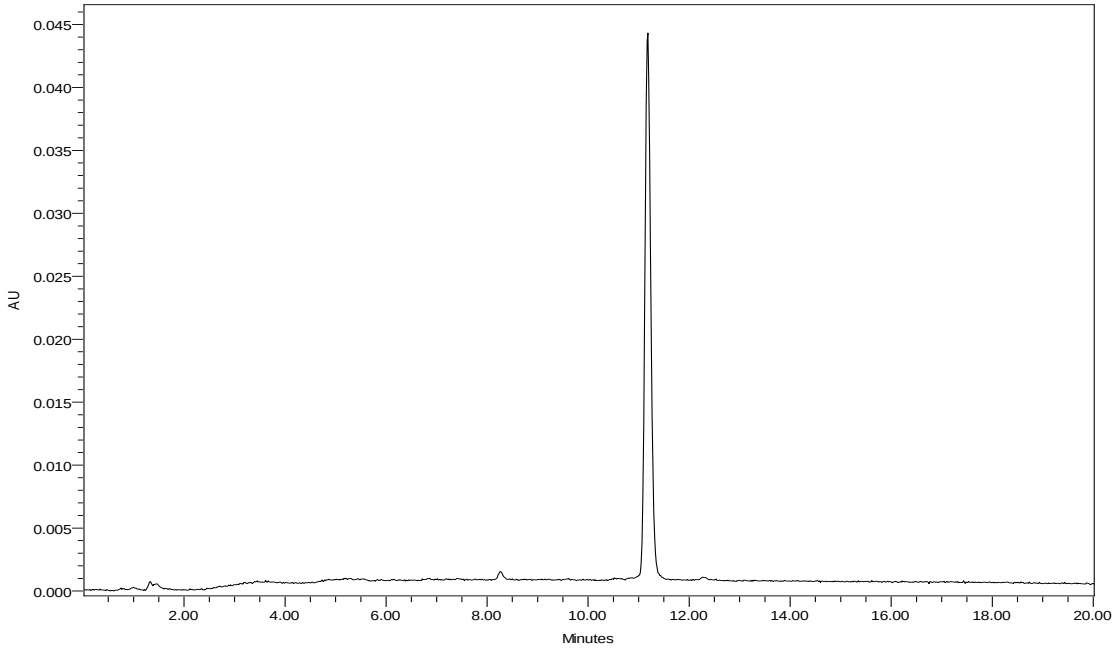
16.00
18.00
20.00
Minutes

rON-11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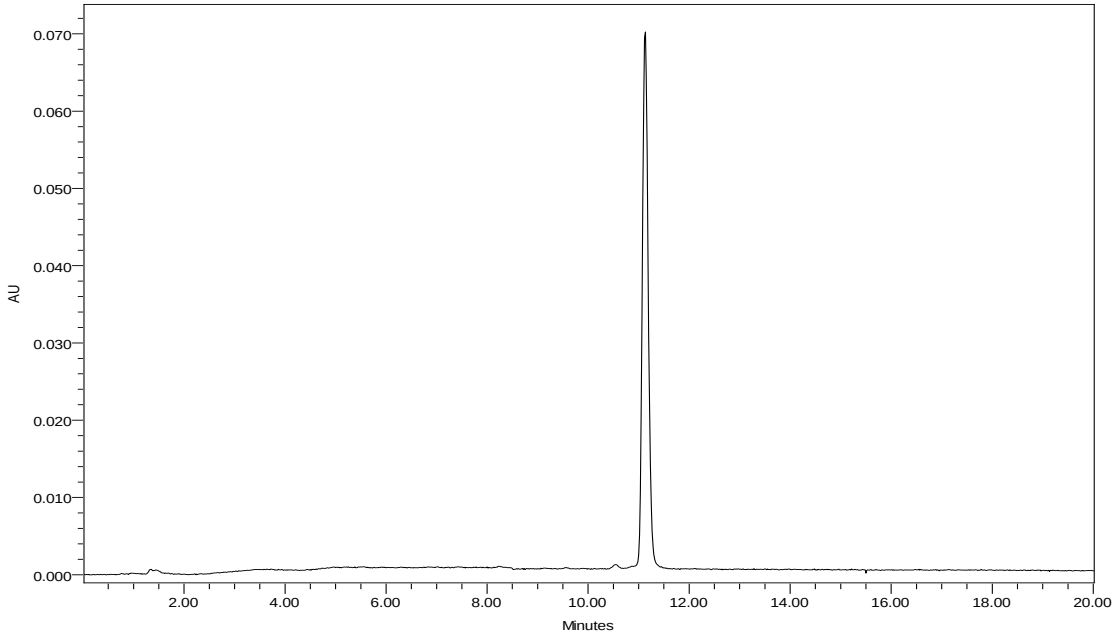
Minutes

rON-12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AU
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6.00
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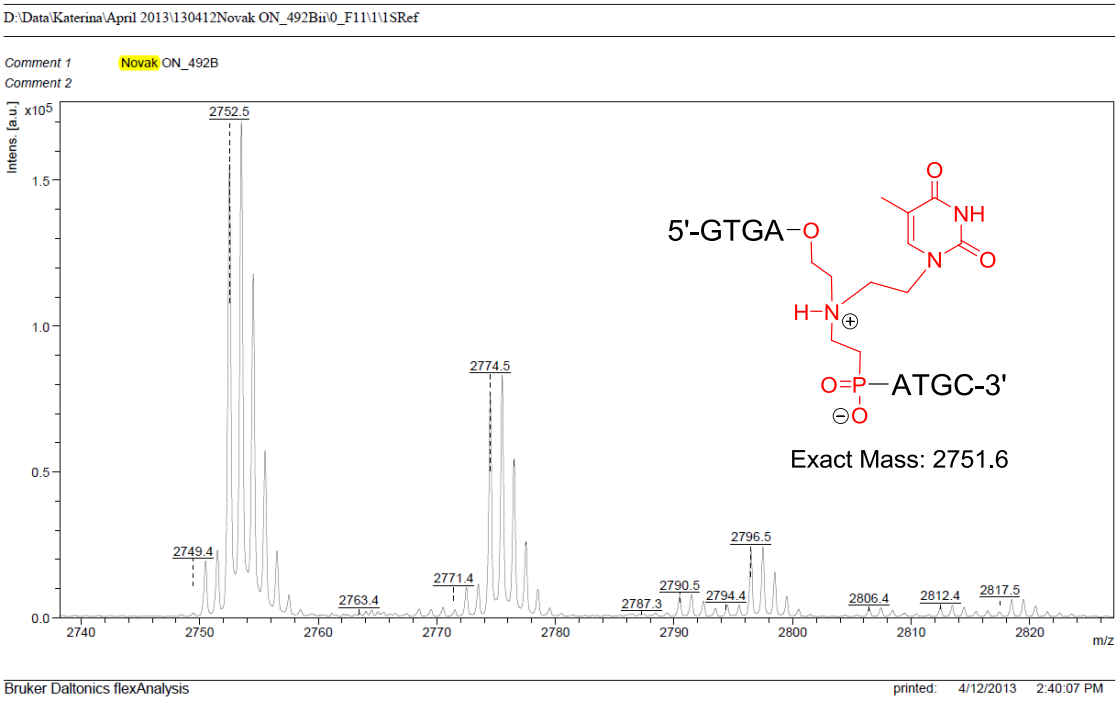
12.00
14.00
16.00
18.00
20.00
Minutes

MALDI TOF MS of the prepared modified nonamers

dON-2



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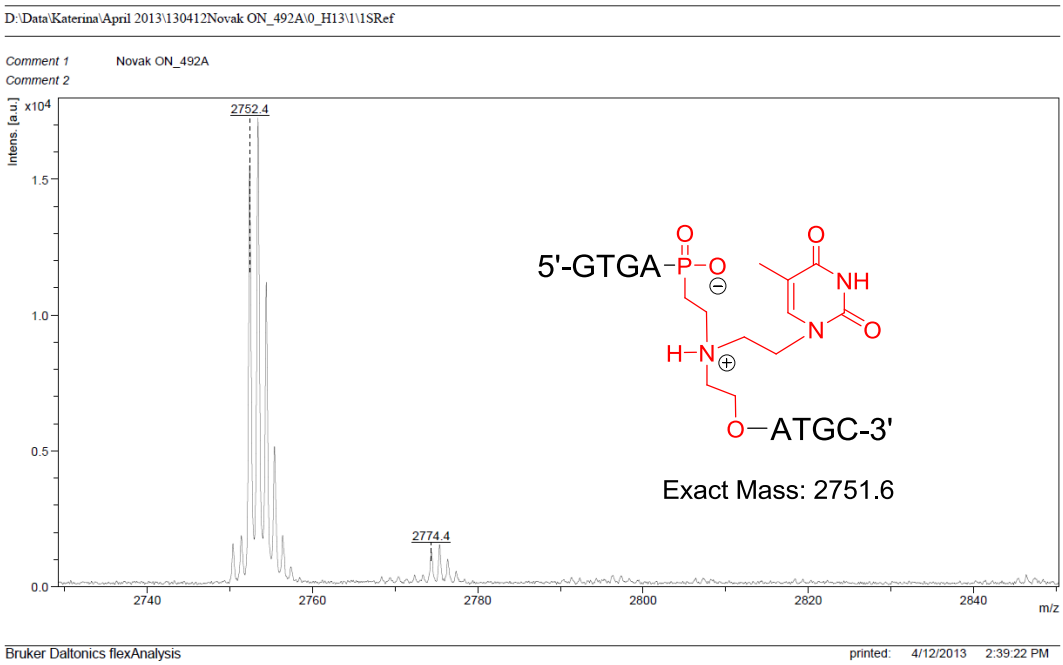

dON-5

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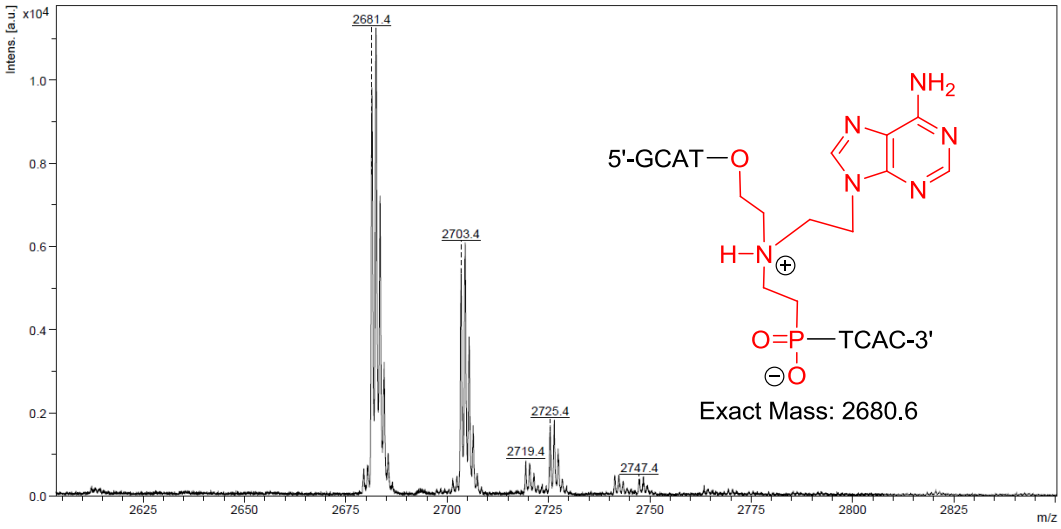
Comment 2



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dON-6

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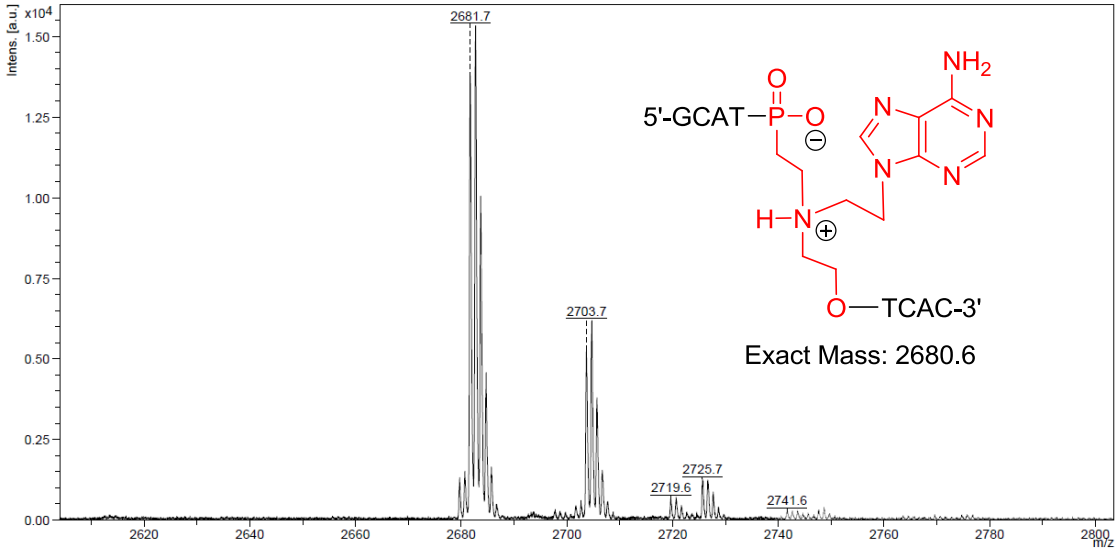
Comment 2 HPA/PA/vinan, monoisotopic mass



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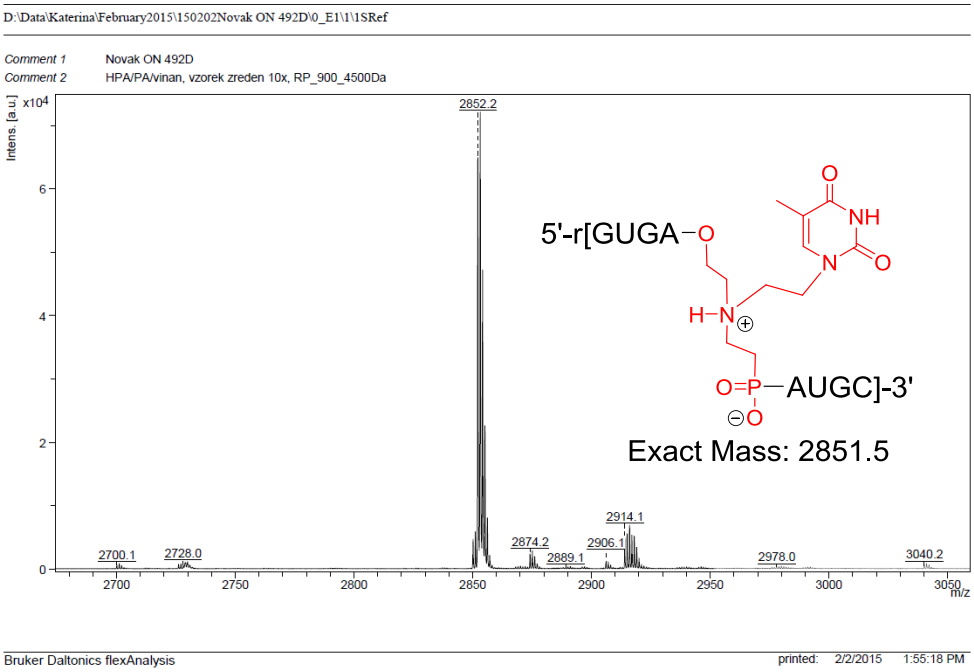
rON-8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2914.1
2874.2
2906.1
2689.1
2700.1
2728.0
2978.0
3040.2
m/z
5'-r[GUGA
AUGC]-3'
Exact Mass: 2851.5
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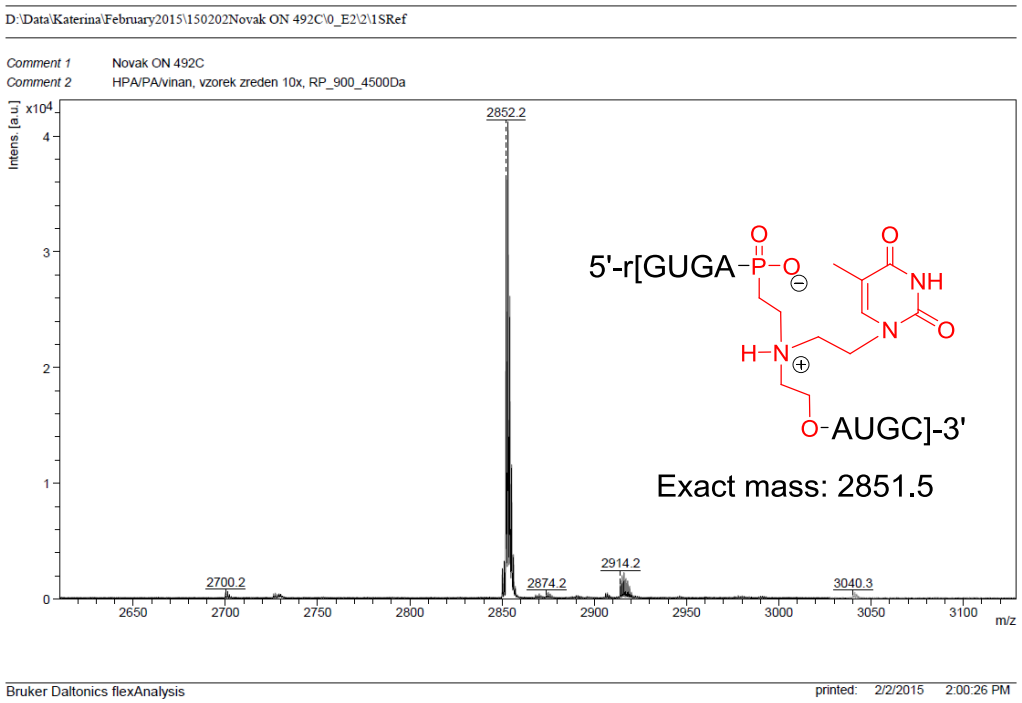
rON-9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Comment 2 HPA/PA/vinan, vzorek zreden 10x, RP_900_4500Da
Intens. [a.u.] x10^4
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2700.2
2874.2
3040.3
m/z
5'-r[GUGA
AUGC]-3'
Exact mass: 2851.5
Bruker Daltonics flexAnalysis
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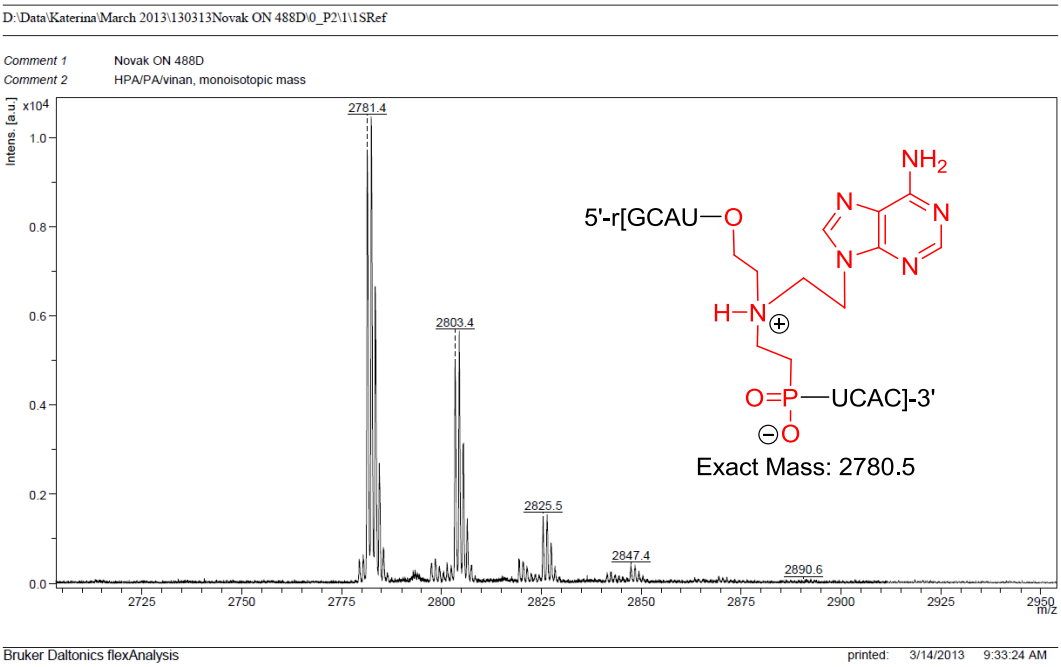
rON-11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2847.4
2890.6
5'-r[GCAU—O
H—N
O=P—UCAC]-3'
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NH2
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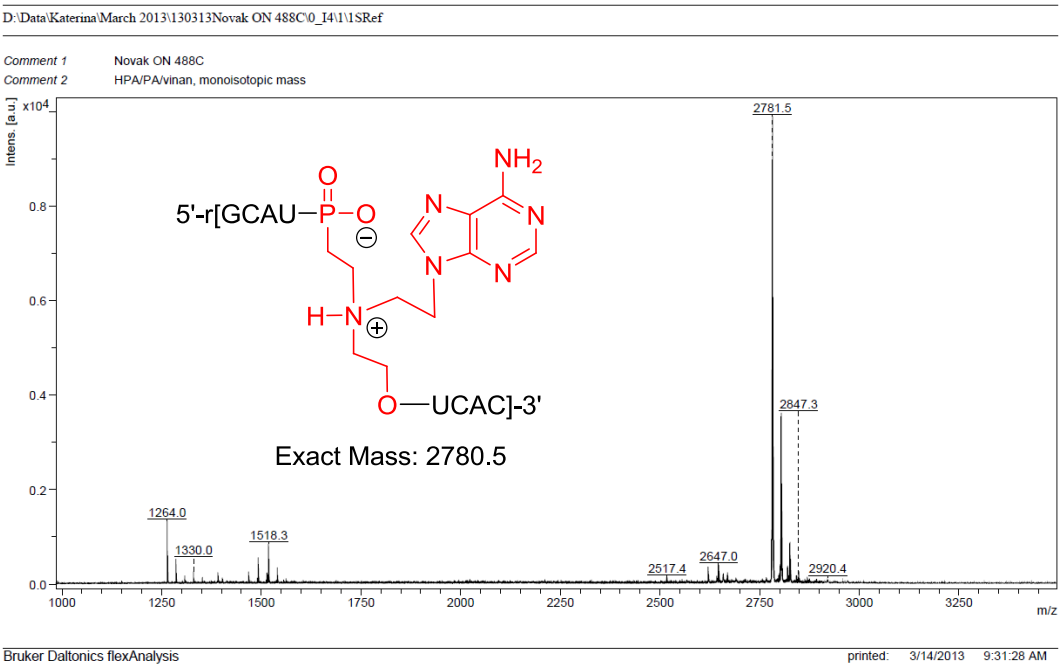
rON-12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2847.3
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2647.0
2920.4
5'-r[GCAU—P—O
H—N
O—UCAC]-3'
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NH2
Exact Mass: 2780.5
m/z
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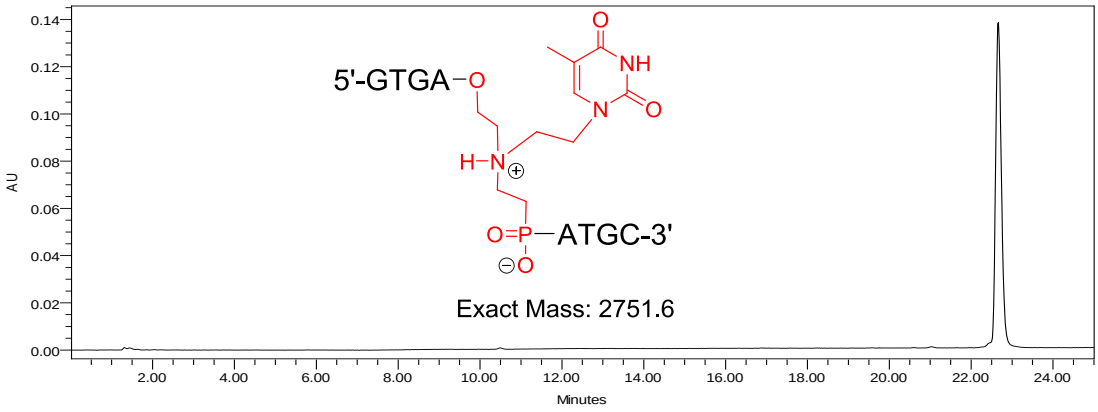
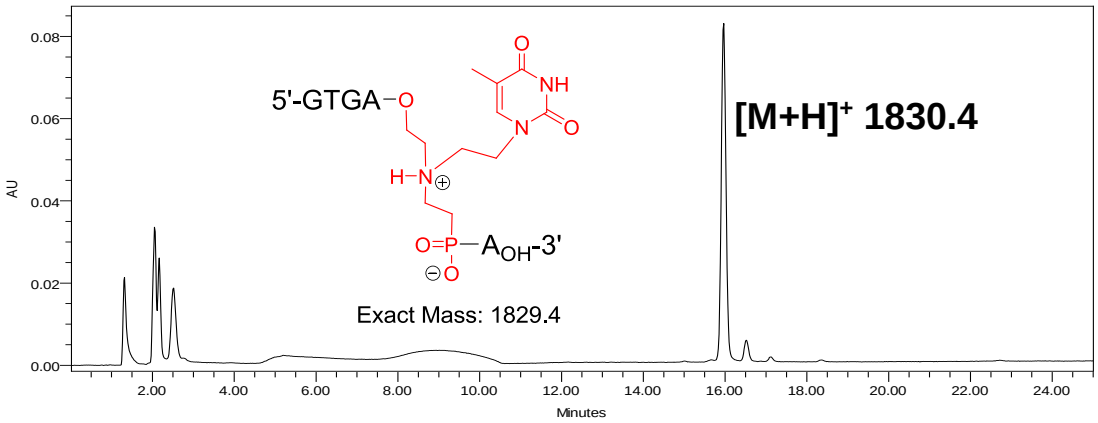
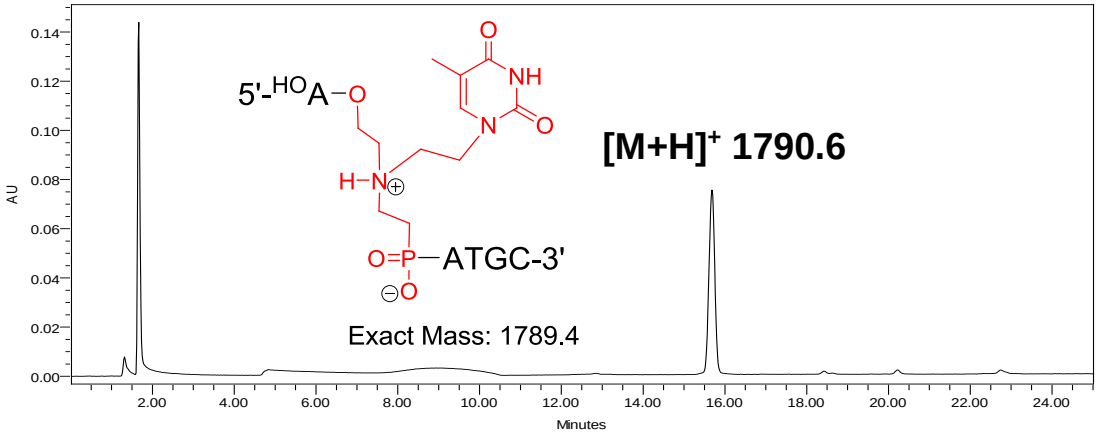
IE HPLC profile of d**ON-2**



IE HPLC profile of the cleavage of d**ON-2** by PDase I



IE HPLC profile of the cleavage of d**ON-2** by PDase II



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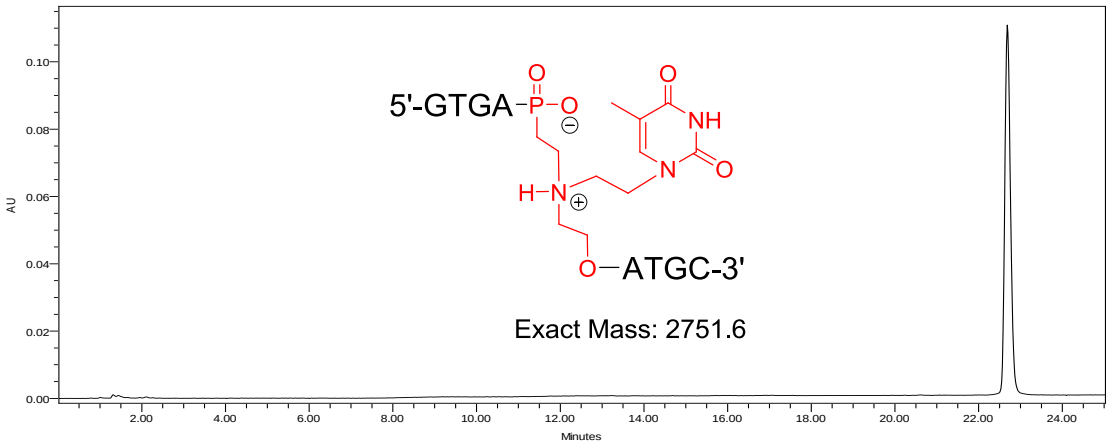
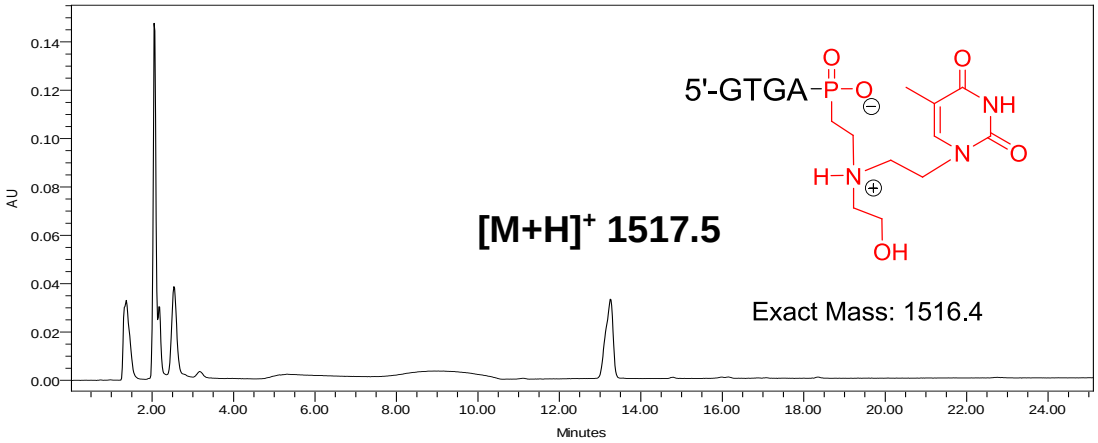


IE HPLC profile of the cleavage of d**ON-3** by PDase I



IE HPLC profile of the cleavage of d**ON-3** by PDase II

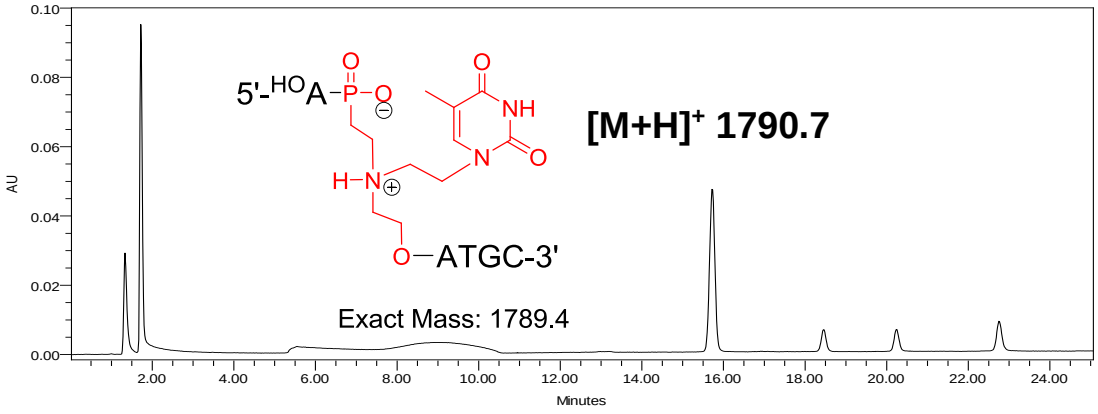


Table 1. Comparison of T_m values and thermodynamic parameters of complexes of unmodified and modified oligonucleotides with central nucleoside phosphonate unit.

Entry	Sequence 5'→3'	$T_m/\Delta T_m$ [°C] complement		ΔG_{293} [kJ/mol] complement		ΔH [kJ/mol] complement		ΔS [J/mol/K] complement	
		DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA
dON-1	**d(GTG ATA TGC)**	36.9/0.0	34.5/0.0	–49.3	–43.7	–323	–272	–934	–779
dON-2	**d(GTG AT^A TGC)**	27.0/–9.9	24.8/–9.7	–38.6	–36.4	–298	–281	–885	–833
dON-3	**d(GTG A^TA TGC)**	23.2/–13.7	24.2/–10.3	–36.1	–35.1	–276	–262	–818	–772
dON-4	**d(GCA TAT CAC)**	36.9/0.0	34.9/0.0	–49.3	–46.9	–323	–302	–934	–870
dON-5	**d(GCA TA^T CAC)**	29.6/–7.3	28.5/–6.4	–41.5	–41.2	–315	–320	–931	–950
dON-6	**d(GCA T^AT CAC)**	27.6/–9.3	27.3/–7.6	–38.8	–39.1	–295	–326	–874	–979
rON-7	**r(GUG ATA UGC)**	34.9/0.0	46.1/0.0	–46.9	–57.2	–302	–344	–870	–978
rON-8	**r(GUG AT^A UGC)**	23.9/–11.0	33.0/–13.1	–35.5	–45.0	–289	–326	–866	–959
rON-9	**r(GUG A^TA UGC)**	19.3/–15.6	28.5/–17.6	–28.5	–40.0	–287	–295	–883	–872
rON-10	**r(GCA UAU CAC)**	34.5/0.0	46.1/0.0	–49.3	–57.2	–323	–344	–934	–978
rON-11	**r(GCA UA^U CAC)**	28.4/–6.1	36.5/–9.6	–39.4	–47.9	–296	–340	–876	–997
rON-12	**r(GCA U^AU CAC)**	22.0/–12.5	33.3/–12.8	–34.9	–46.9	–275	–361	–819	–1070

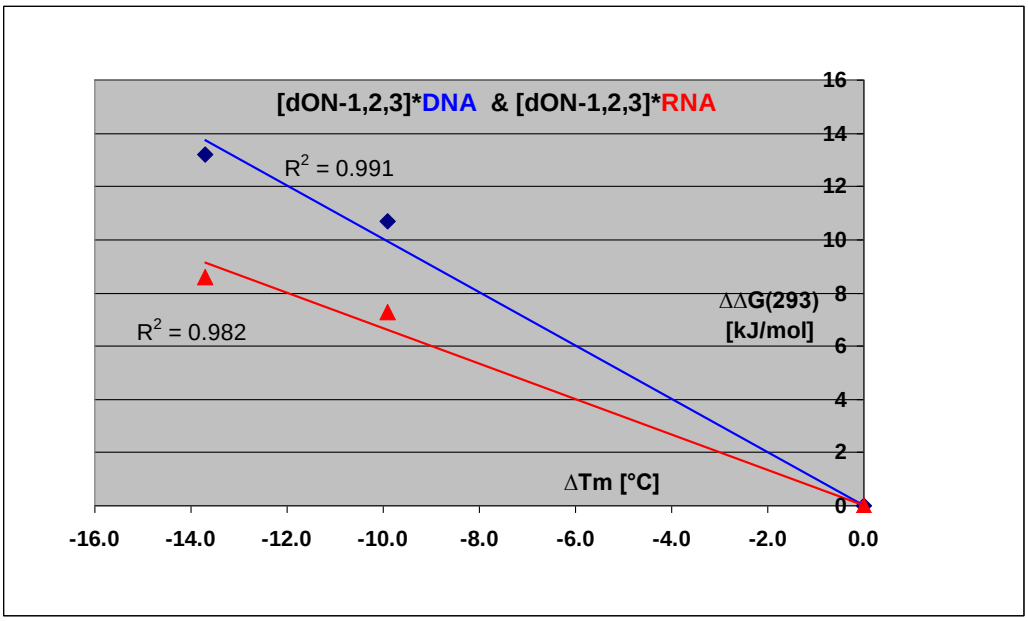
All thermodynamic data are an average of five determination using Varian Cary Bio-100 built-in software; standard deviation varied ±7%. **T^** (**A^**) and **^T** (**^A**) mean acyclic nucleoside phosphonate units with 3'- and 5'-phosphonate-oriented moiety, respectively. **ON-1, ON-4, ON-7,** and **ON-10** are unmodified nonamers.

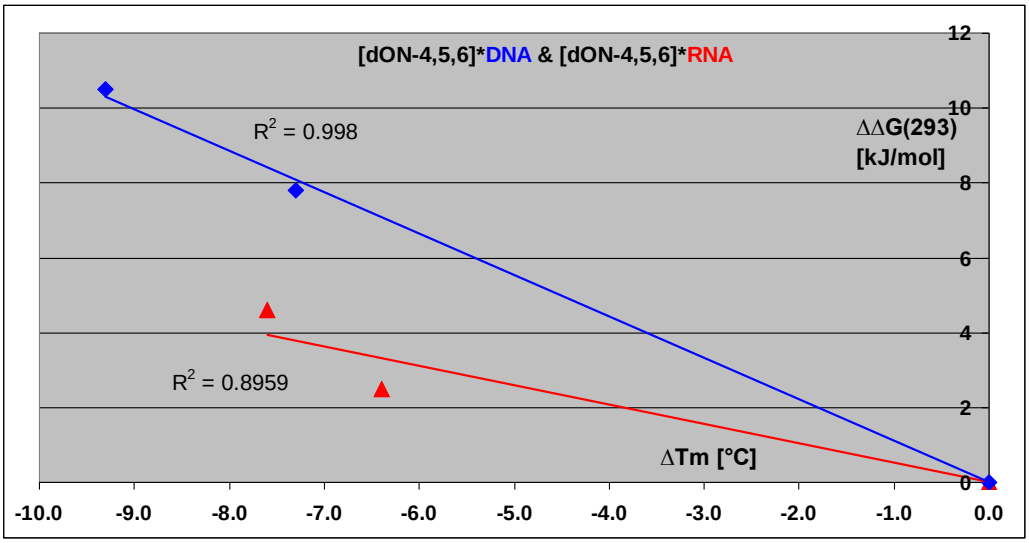
Table 2. Gibbs free energy $\Delta\Delta G_{293}$ *vs.* ΔT_m values.

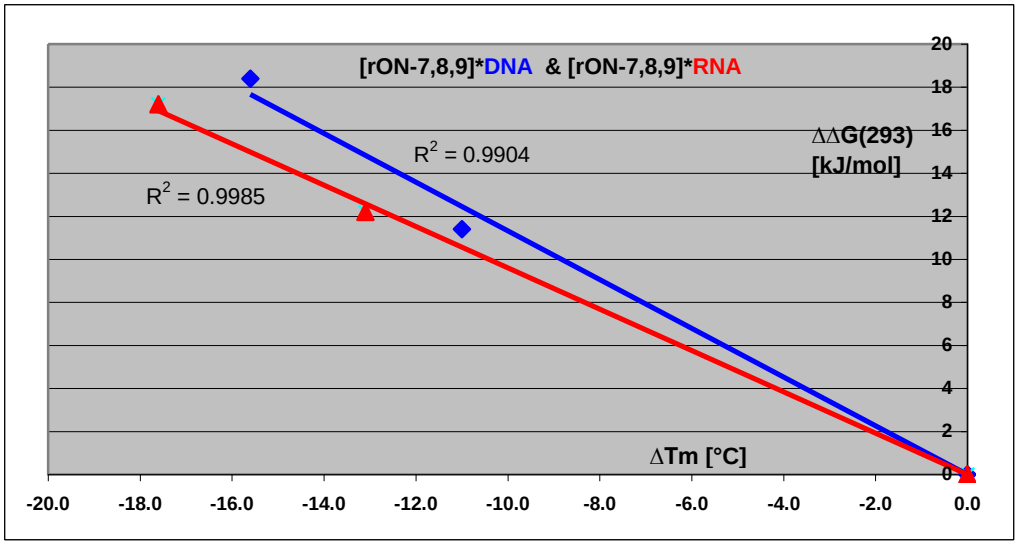
Entry	Sequence 5'→3'	ΔT_m [°C] complement		$\Delta\Delta G_{293}$[a] [kJ/mol] complement	
		DNA	RNA	DNA	RNA
dON-1	**d(GTG ATA TGC)**	0	0	0	0
dON-2	**d(GTG AT^A TGC)**	–9.9	–9.7	10.7	7.3
dON-3	**d(GTG A^TA TGC)**	–13.7	–10.3	13.2	8.6
dON-4	**d(GCA TAT CAC)**	0.0	0.0	0	0
dON-5	**d(GCA TA^T CAC)**	–7.3	–6.4	7.8	2.5
dON-6	**d(GCA T^AT CAC)**	–9.3	–7.6	10.5	4.6
rON-7	**r(GUG ATA UGC)**	0	0	0	0
rON-8	**r(GUG AT^A UGC)**	–11.0	–13.1	11.4	12.2
rON-9	**r(GUG A^TA UGC)**	–15.6	–17.6	18.4	17.2
rON-10	**r(GCA UAU CAC)**	0	0	0	0
rON-11	**r(GCA UA^U CAC)**	–6.1	–9.6	7.5	9.3
rON-12	**r(GCA U^AU CAC)**	–12.5	–12.8	12	10.3

[a] difference in Gibbs free energies between the modified and natural duplexes; ($\Delta\Delta G_{293} = {}^{modif}\Delta G_{293} - {}^{natural}\Delta G_{293}$)

Graphical imaging of data from Table 2.






[rON-7,8,9]*DNA & [rON-7,8,9]*RNA
R2 = 0.9904
R2 = 0.9985
ΔΔG(293) [kJ/mol]
ΔTm [°C]
20
18
16
14
12
10
8
6
4
2
0
-20.0
-18.0
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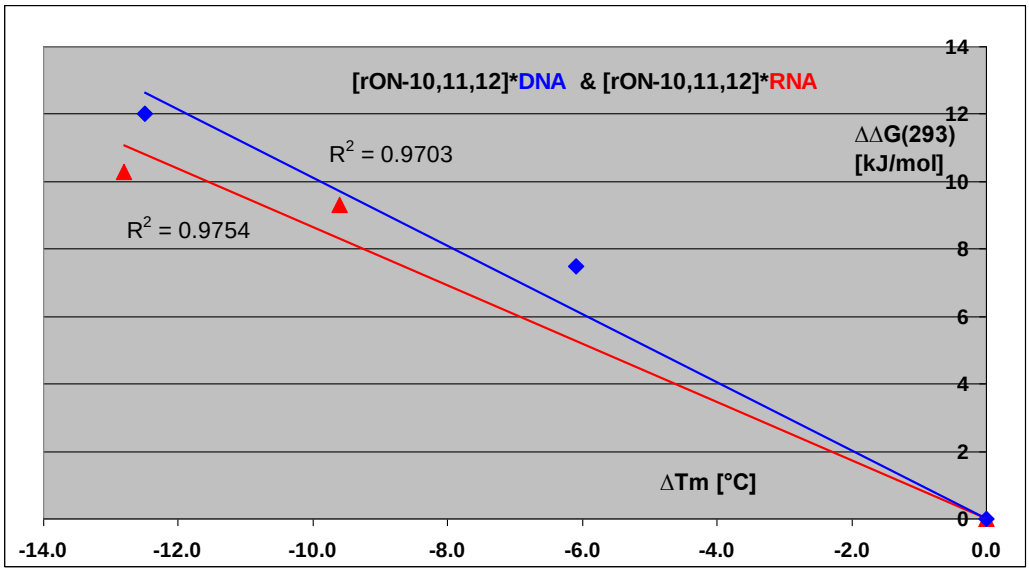
[rON-10,11,12]*DNA & [rON-10,11,12]*RNA
R2 = 0.9703
R2 = 0.9754
ΔΔG(293) [kJ/mol]
ΔTm [°C]
14
12
10
8
6
4
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-14.0
-12.0
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-6.0
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-2.0
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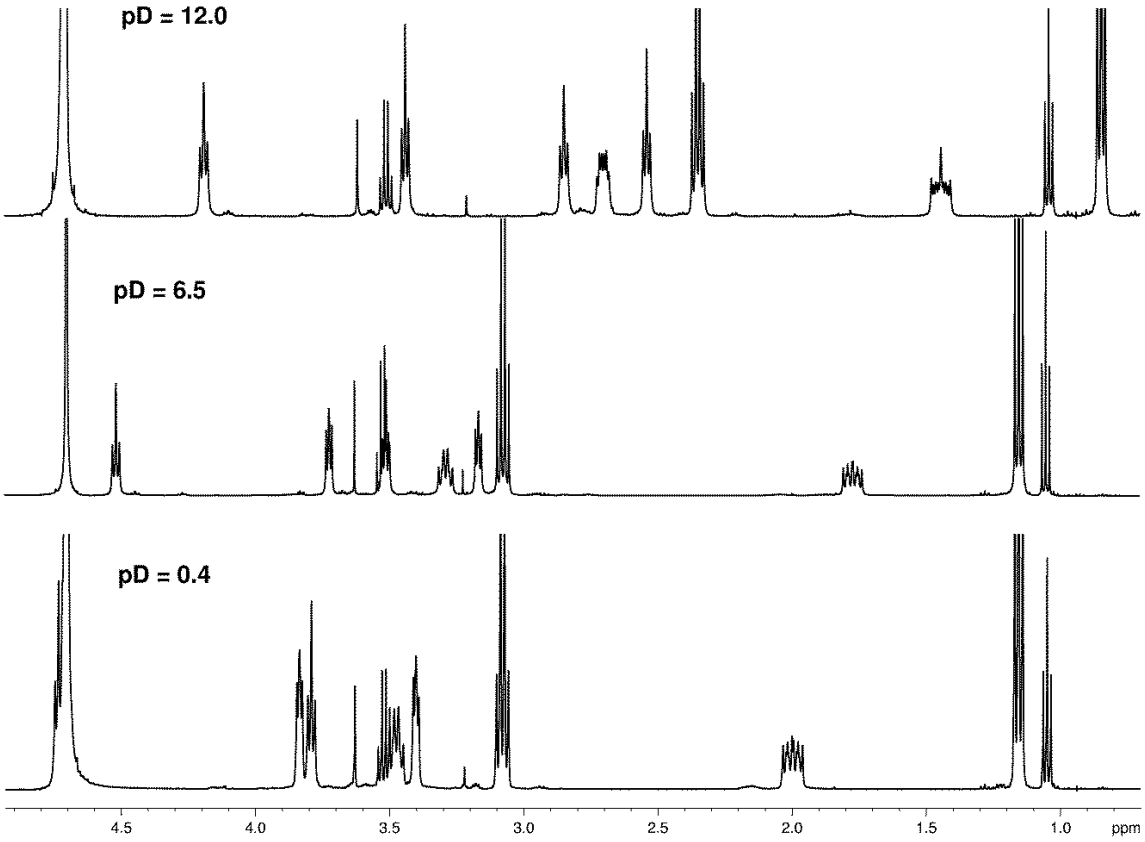
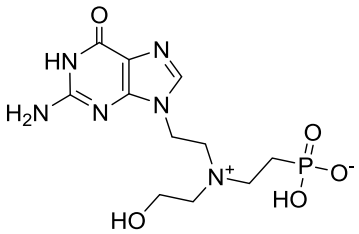
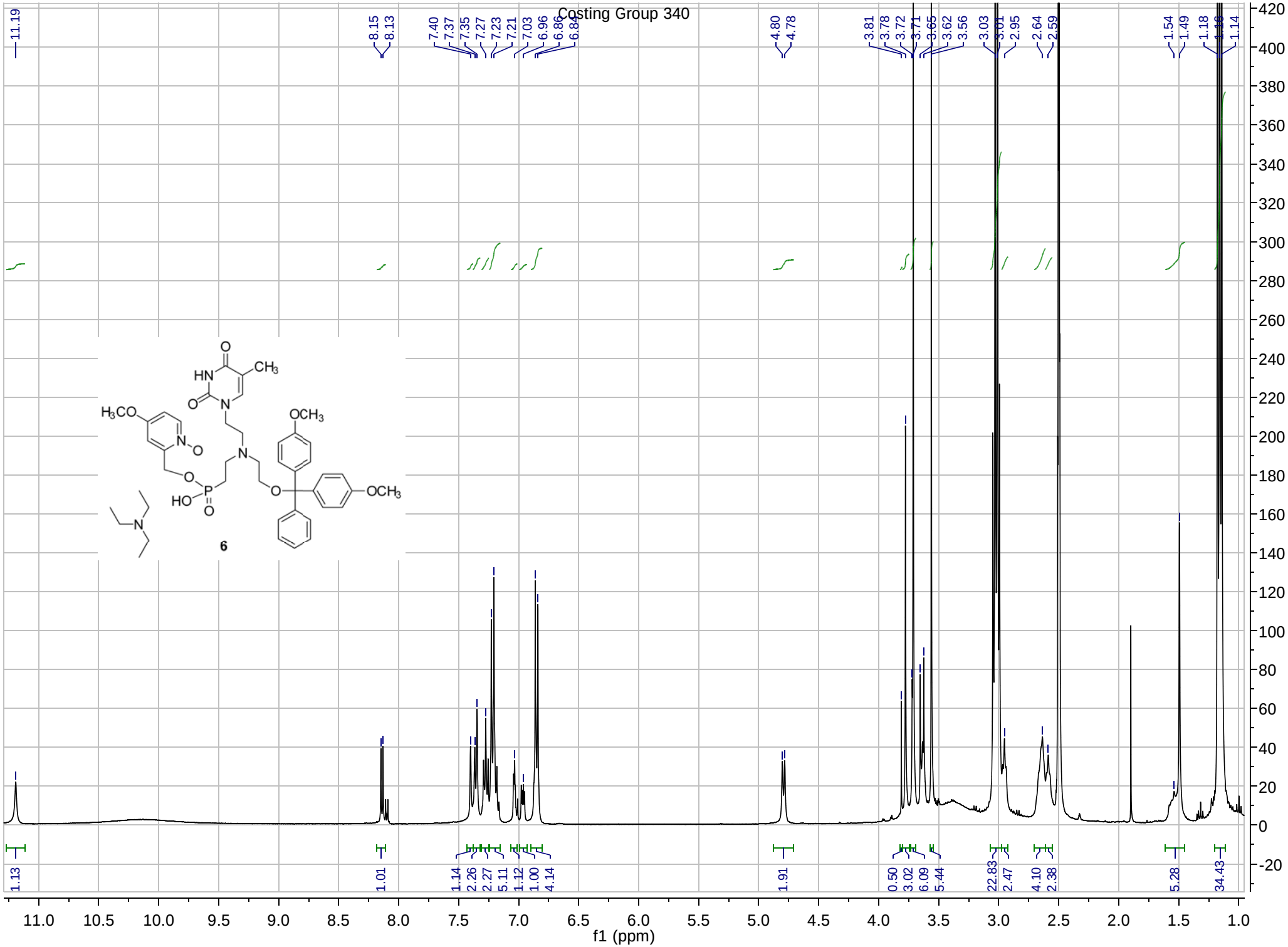


Fig. 4. Aliphatic part of ^{1}H NMR spectra of **A-azaANP** measured at different pD values.



A-azaANP
(originally isolated as triethylammonium salt)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7.40
7.37
7.35
7.27
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7.21
7.03
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6.84
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3.01
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1.49
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1.16
1.14
1.13
1.01
1.14
2.26
2.27
5.11
1.12
1.00
4.14
1.91
0.50
3.02
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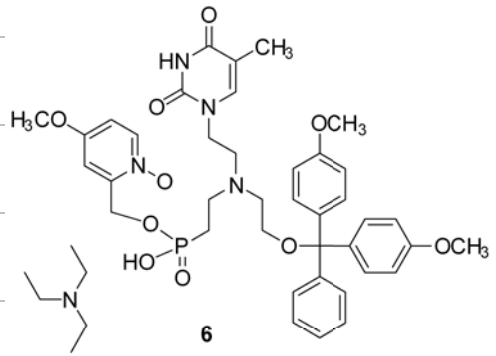
2.47
4.10
2.38
5.28
34.43
f1 (ppm)
6

Costing Group 340



6

164.11
157.86
156.38
150.72
144.84
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135.71
129.45
127.61
127.57
126.44
112.97
112.18
110.12
108.43
107.35
85.39
74.60
74.55
66.23
61.72
59.96
59.92
53.05
52.16
49.57
45.33
21.16
11.50
8.48

4500
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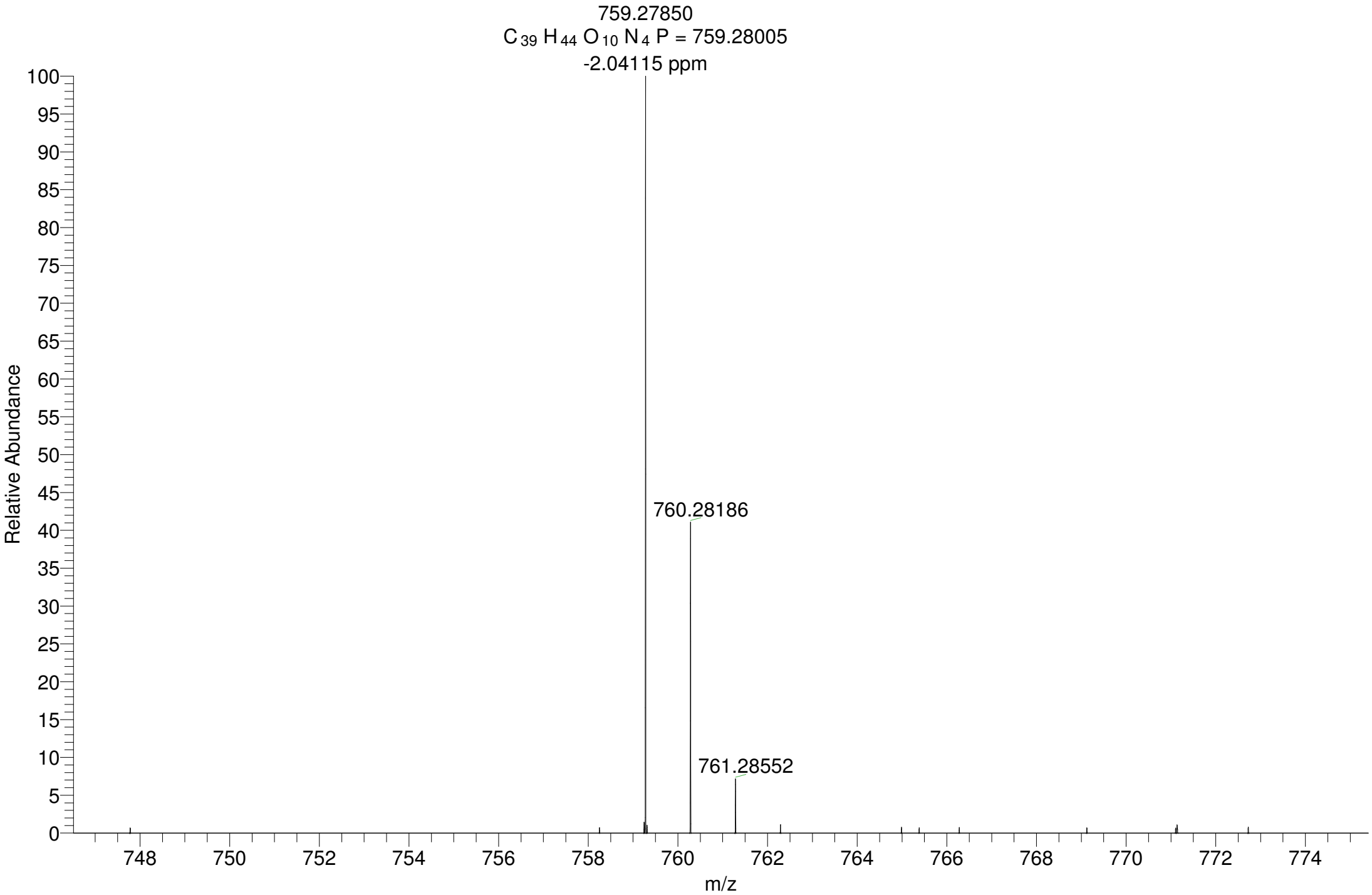
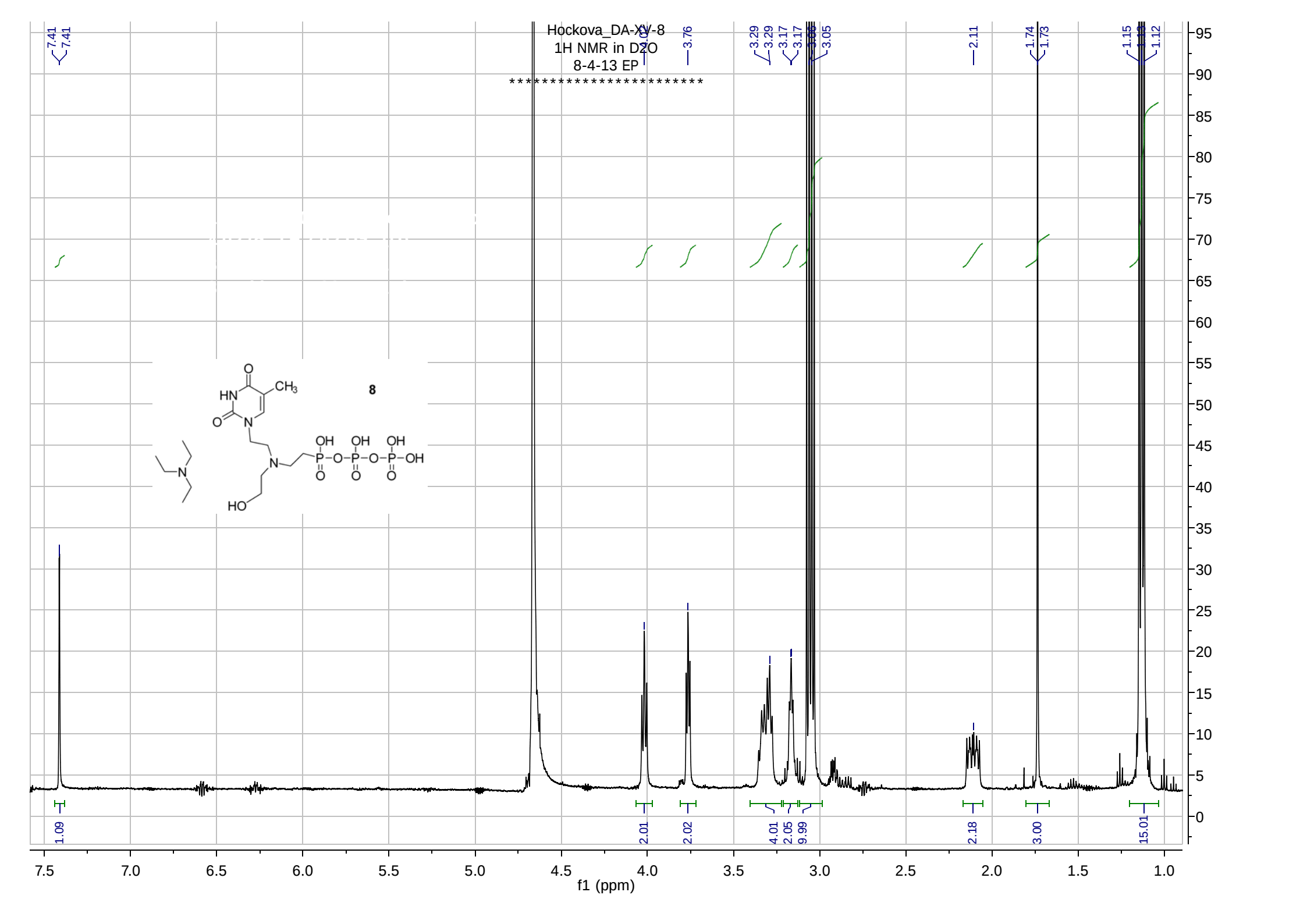
3500
3000
2500
2000
1500
1000
500
0
-500
-1000
-1500
-2000
-2500
-3000

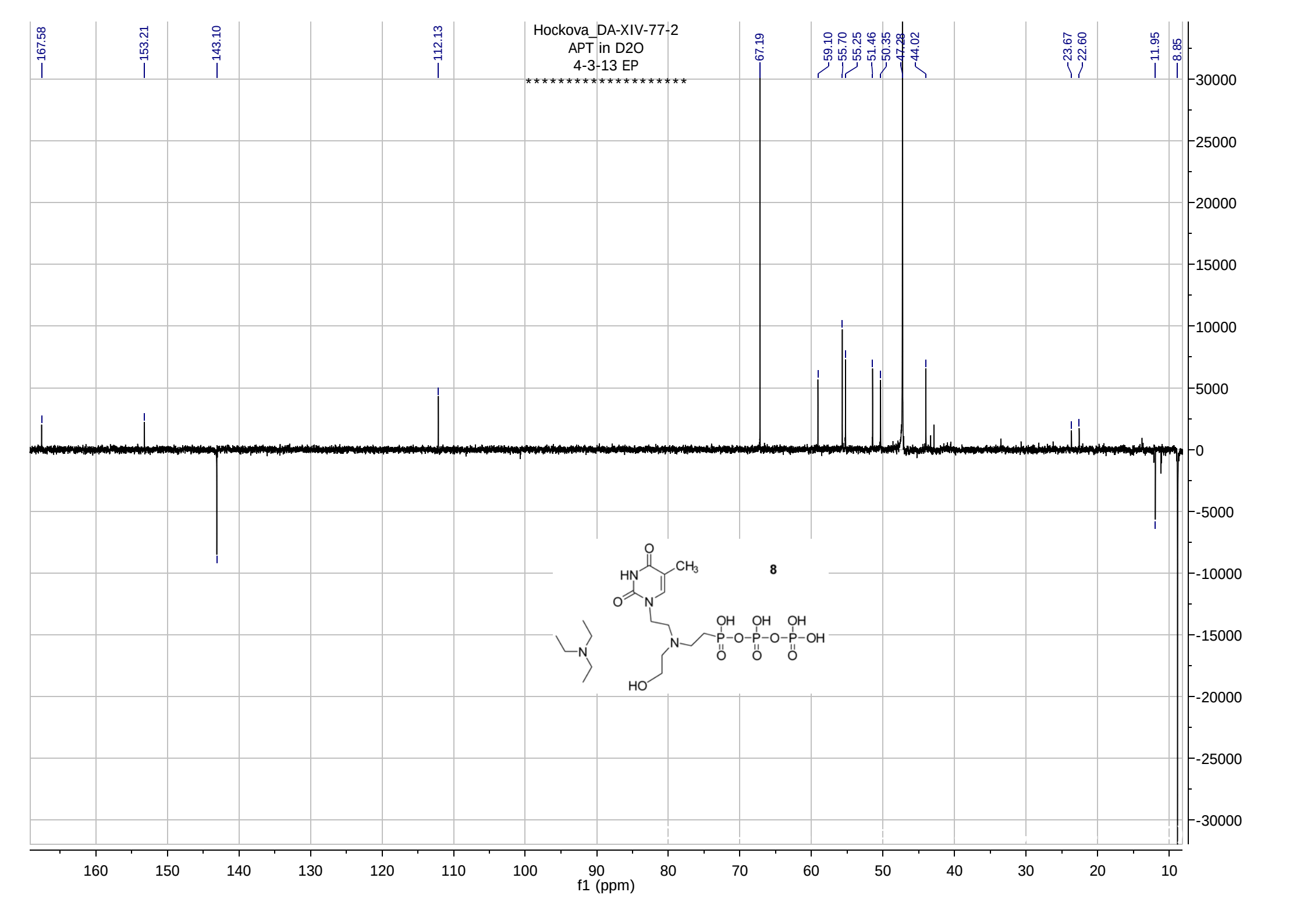
160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10

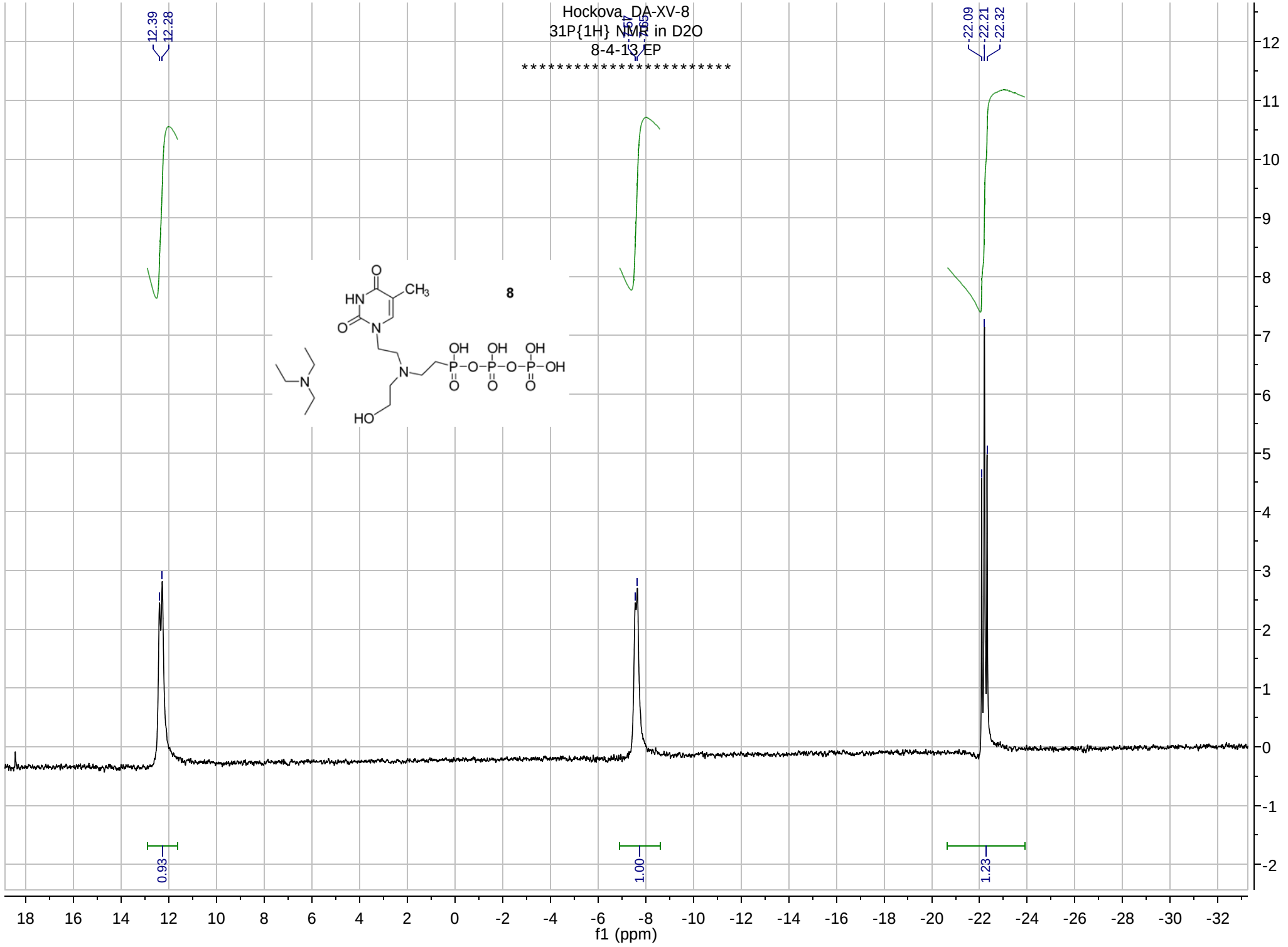
f1 (ppm)

230115servisHR_11 #90 RT: 2.62 AV: 1 NL: 7.55E5
T: FTMS - p ESI Full ms [200.00-2000.00]

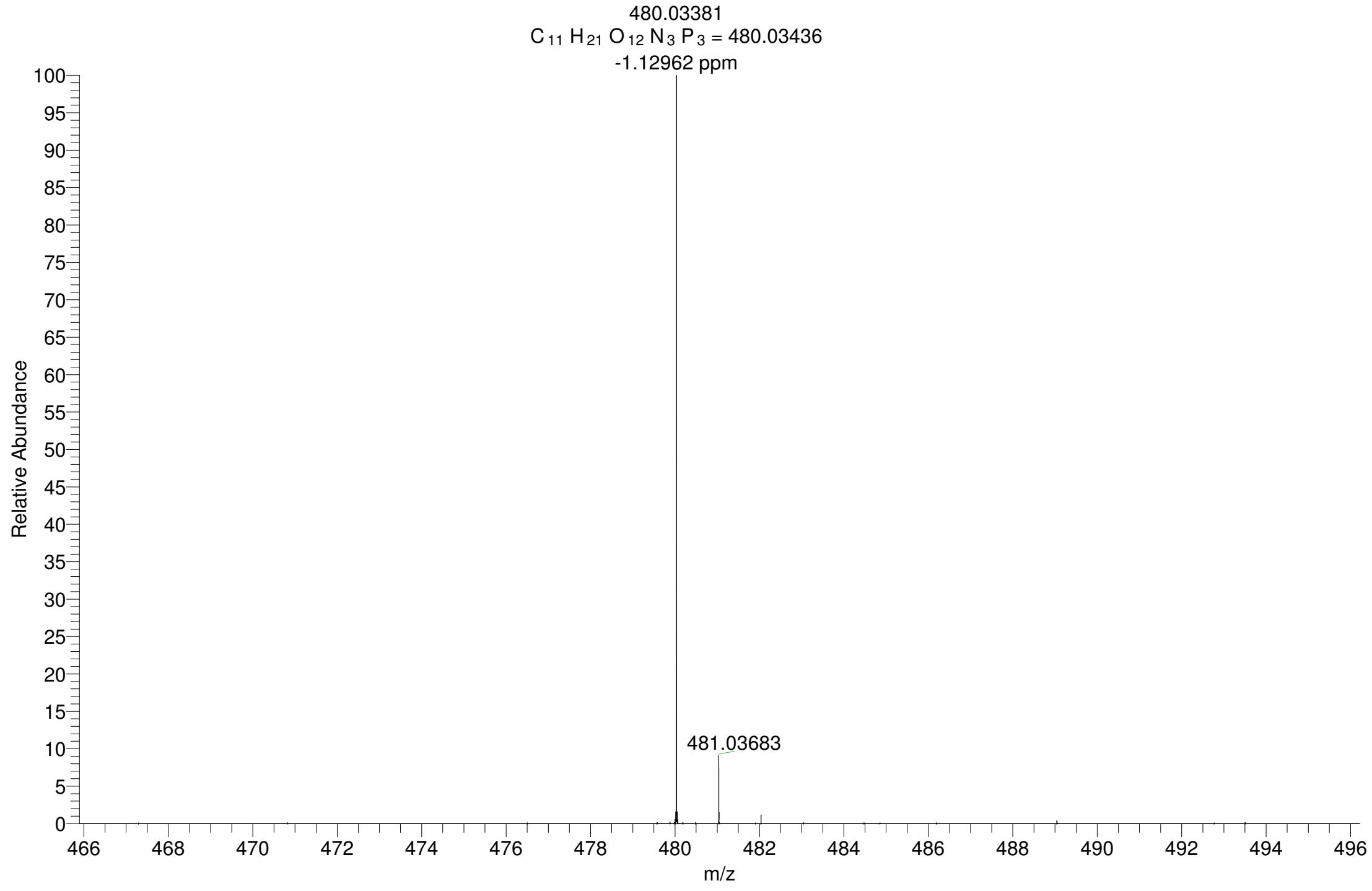


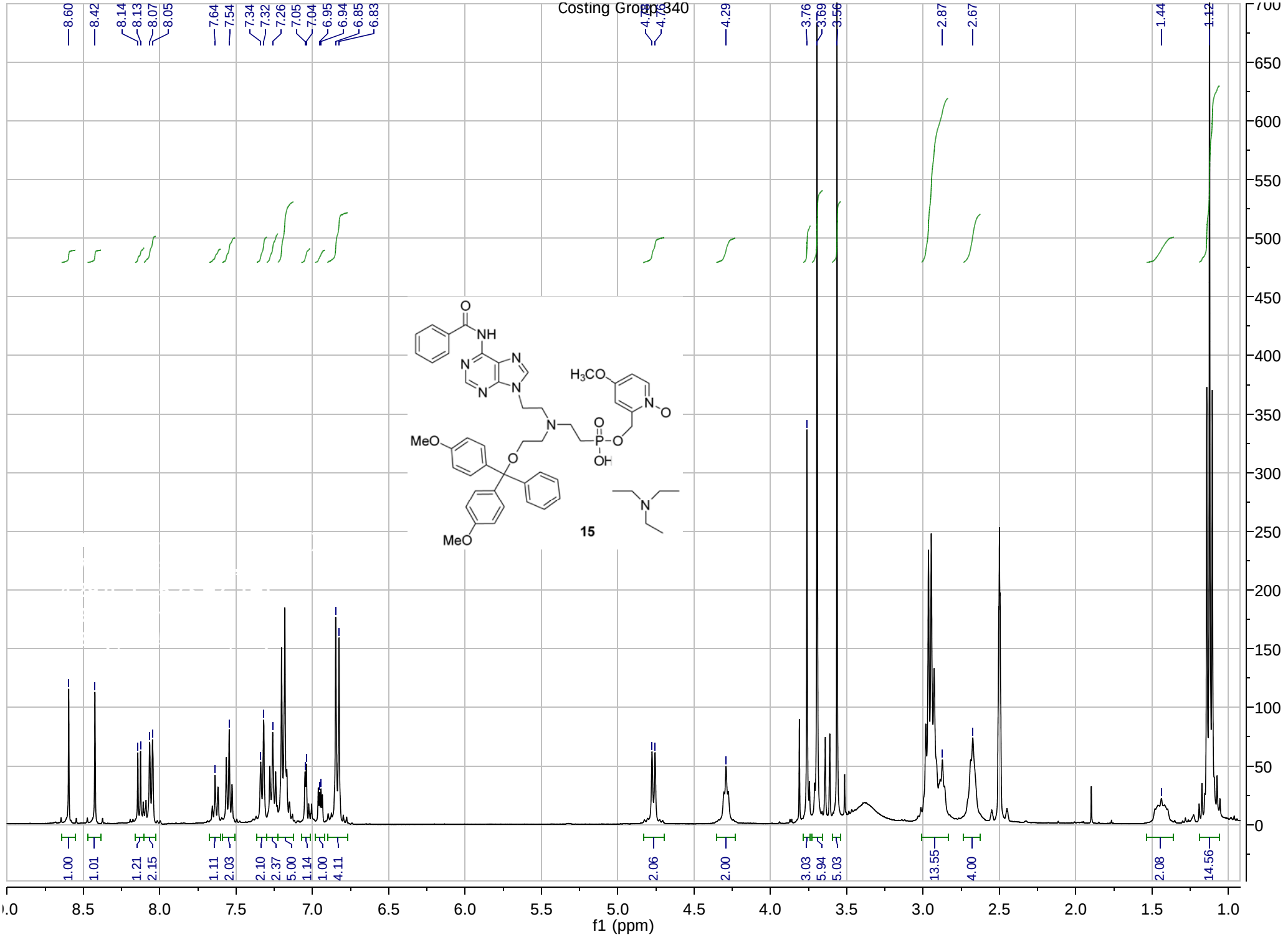

Hockova_DA-XV-8
1H NMR in D2O
8-4-13 EP
****
7.41
7.41
3.76
3.29
3.29
3.17
3.17
3.06
3.05
2.11
1.74
1.73
1.15
1.13
1.12
8
1.09
2.01
2.02
4.01
2.05
9.99
2.18
3.00
15.01
f1 (ppm)

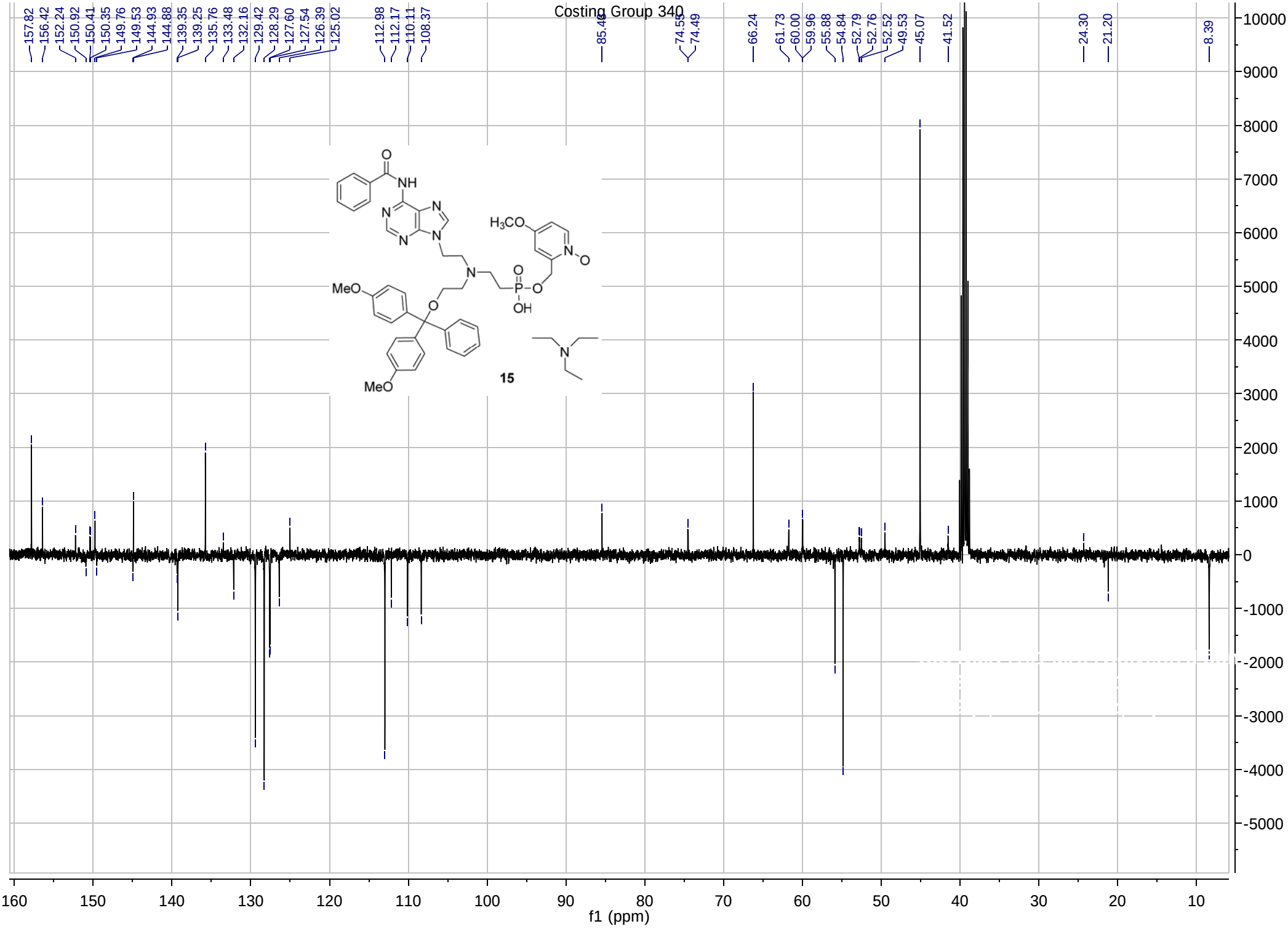

Hockova_DA-XIV-77-2
APT in D2O
4-3-13 EP
****
167.58
153.21
143.10
112.13
67.19
59.10
55.70
55.25
51.46
50.35
47.28
44.02
23.67
22.60
11.95
8.85
8
f1 (ppm)


Hockova_DA-XV-8
31P{1H} NMR in D2O
8-4-13 EP
****
12.39
12.28
7.67
7.65
-22.09
-22.21
-22.32
8
0.93
1.00
1.23
f1 (ppm)

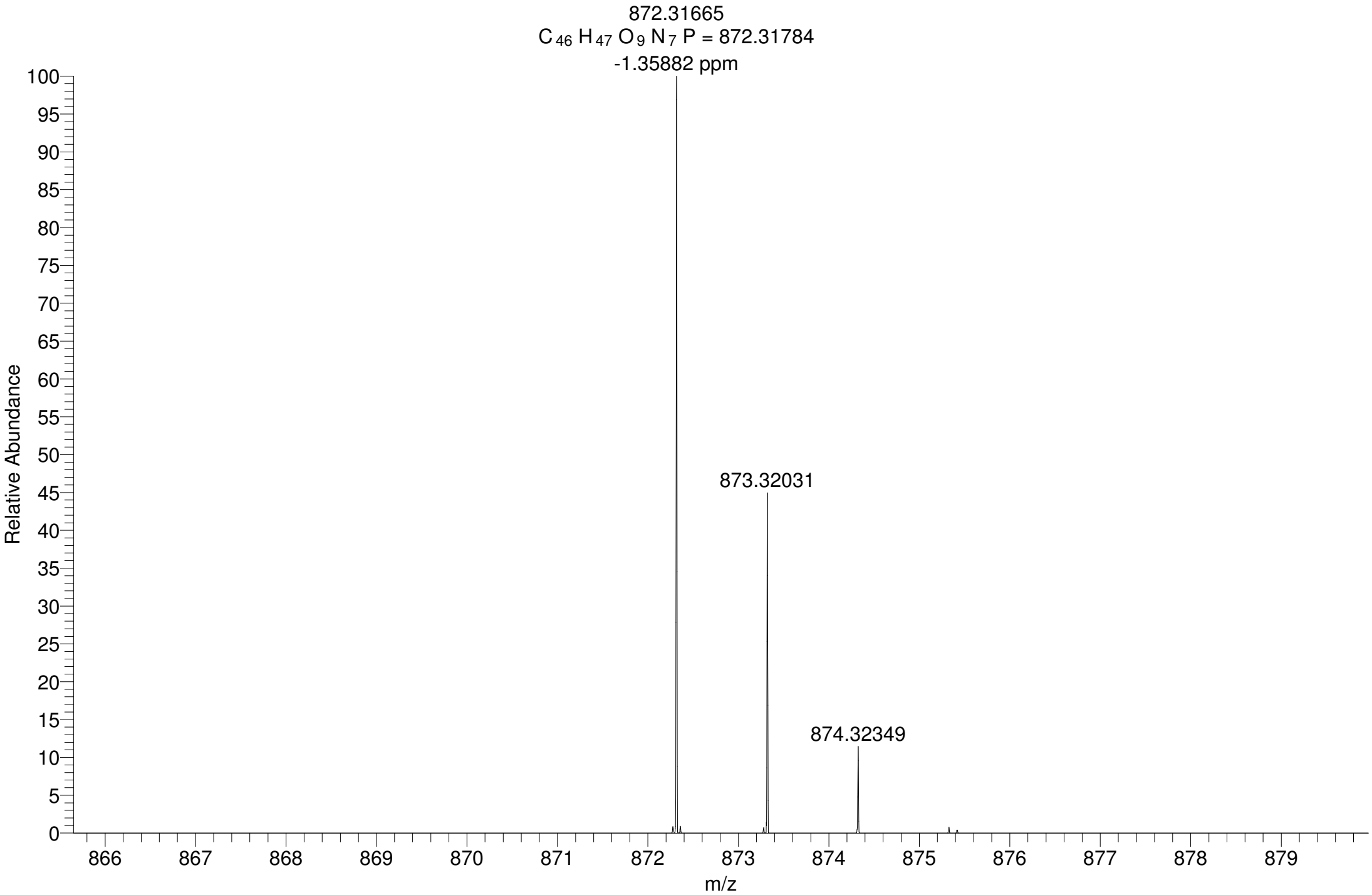
230115servisHR_14 #73 RT: 2.11 AV: 1 NL: 5.16E6
T: FTMS - p ESI Full ms [200.00-2000.00]



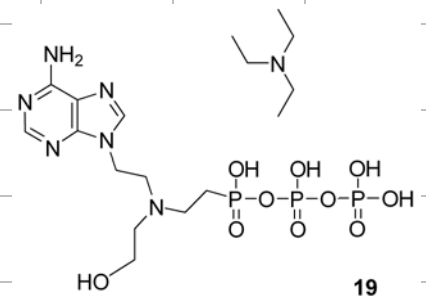

Costing Group 340
8.60
8.42
8.14
8.13
8.07
8.05
7.64
7.54
7.34
7.32
7.26
7.05
7.04
6.95
6.94
6.85
6.83
4.29
3.76
3.69
3.56
2.87
2.67
1.44
1.12
NH
N
O
MeO
H3CO
P
OH
15
1.00
1.01
1.21
2.15
1.11
2.03
2.10
2.37
5.00
1.14
1.00
4.11
2.06
2.00
3.03
5.94
5.03
13.55
4.00
2.08
14.56
f1 (ppm)


Costing Group 340
157.82
156.42
152.24
150.92
150.41
150.35
149.76
149.53
144.93
144.88
139.35
139.25
135.76
133.48
132.16
129.42
128.29
127.60
127.54
126.39
125.02
112.98
112.17
110.11
108.37
85.48
74.53
74.49
66.24
61.73
60.00
59.96
55.88
54.84
52.79
52.76
52.52
49.53
45.07
41.52
24.30
21.20
8.39
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
-2000
-3000
-4000
-5000
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)
O
NH
N
N
N
N
H3CO
N
O
N
O
P
O
OH
MeO
O
MeO
N
15

230115servisHR_12 #70 RT: 2.02 AV: 1 NL: 1.26E6
T: FTMS - p ESI Full ms [200.00-2000.00]



Costing Group 340

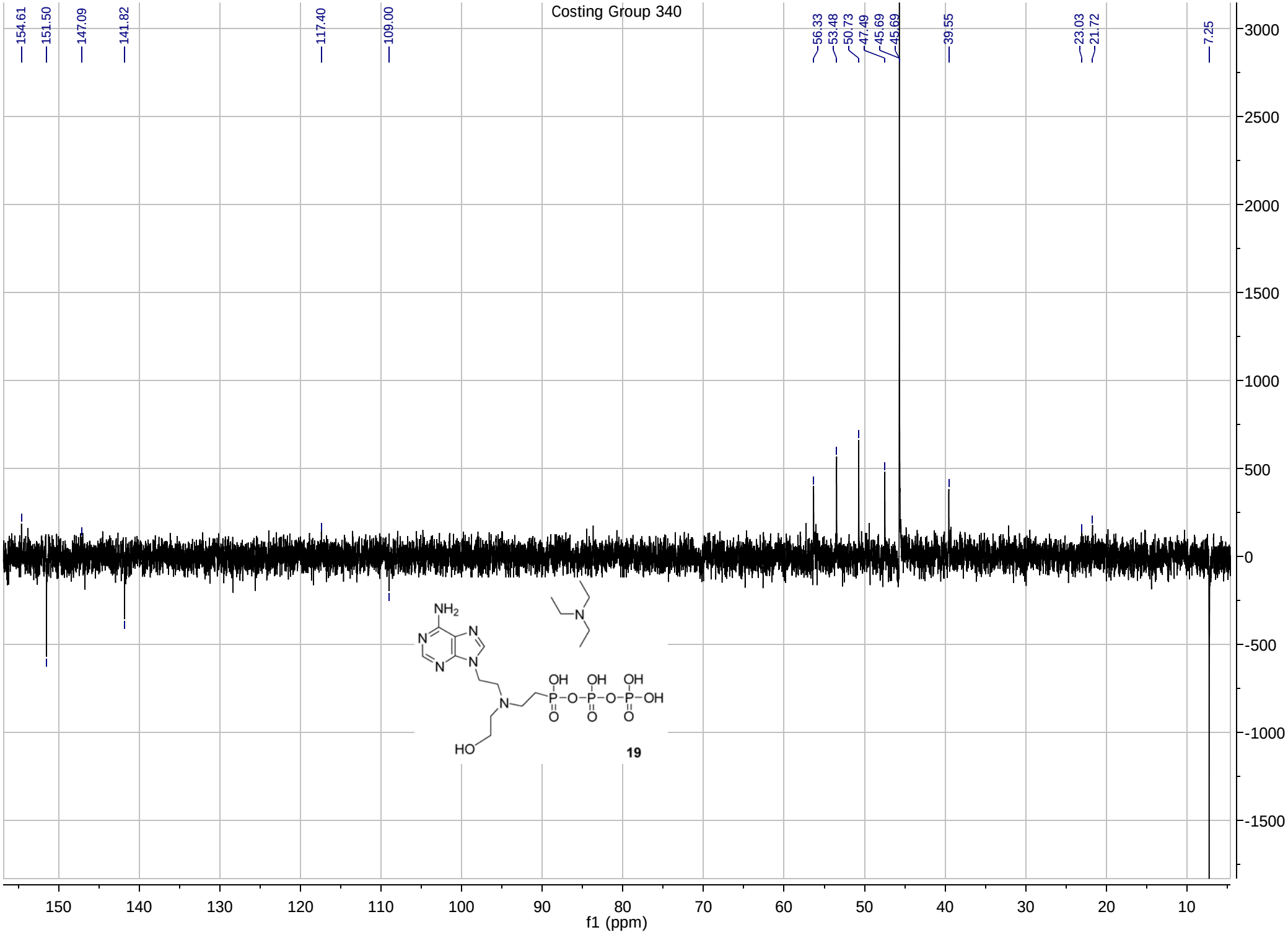


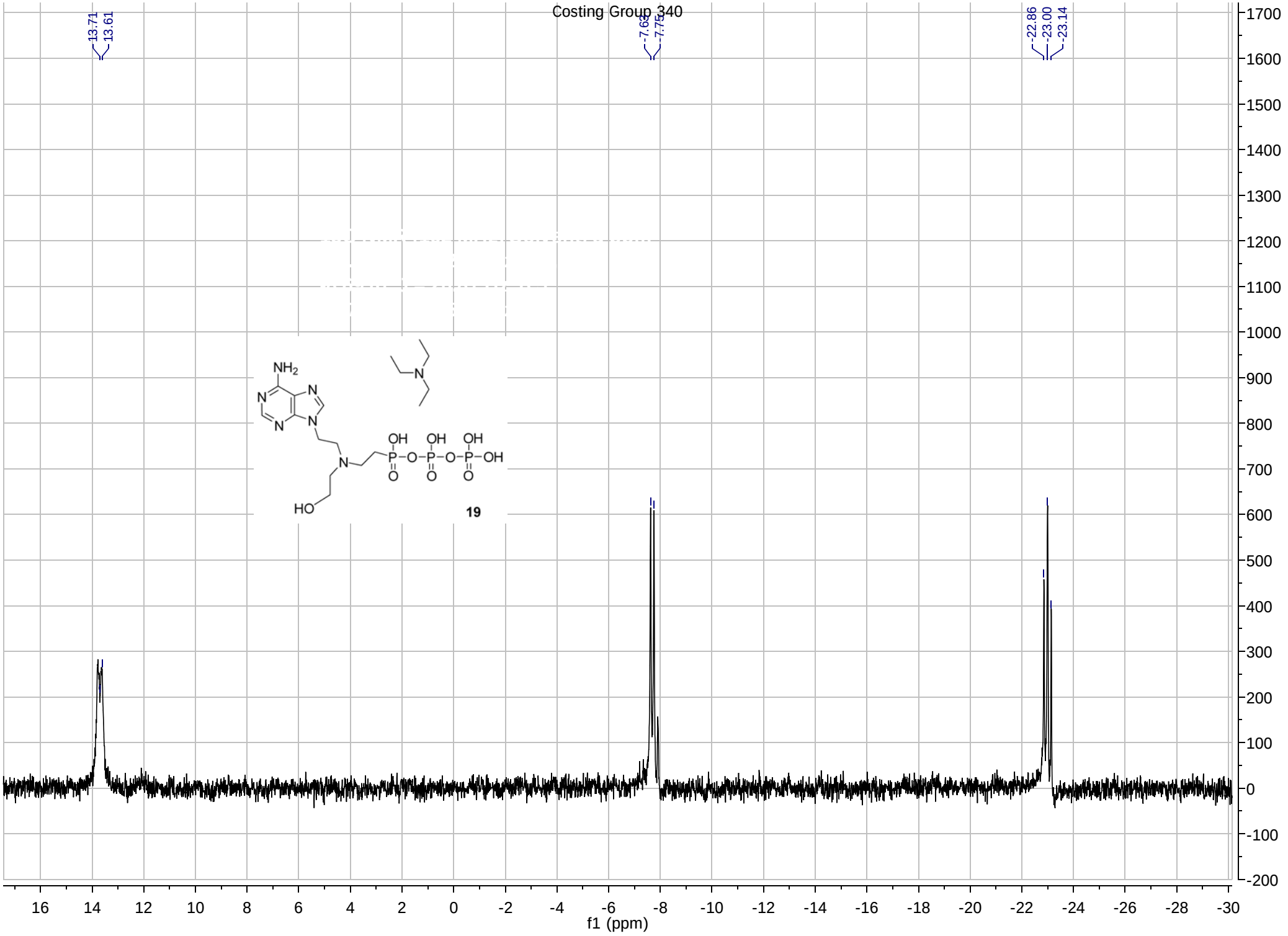
19

8.24
8.22
4.80
4.67
3.83
3.64
3.51
3.49
3.28
3.19
3.17
2.24
2.23
1.28
1.24

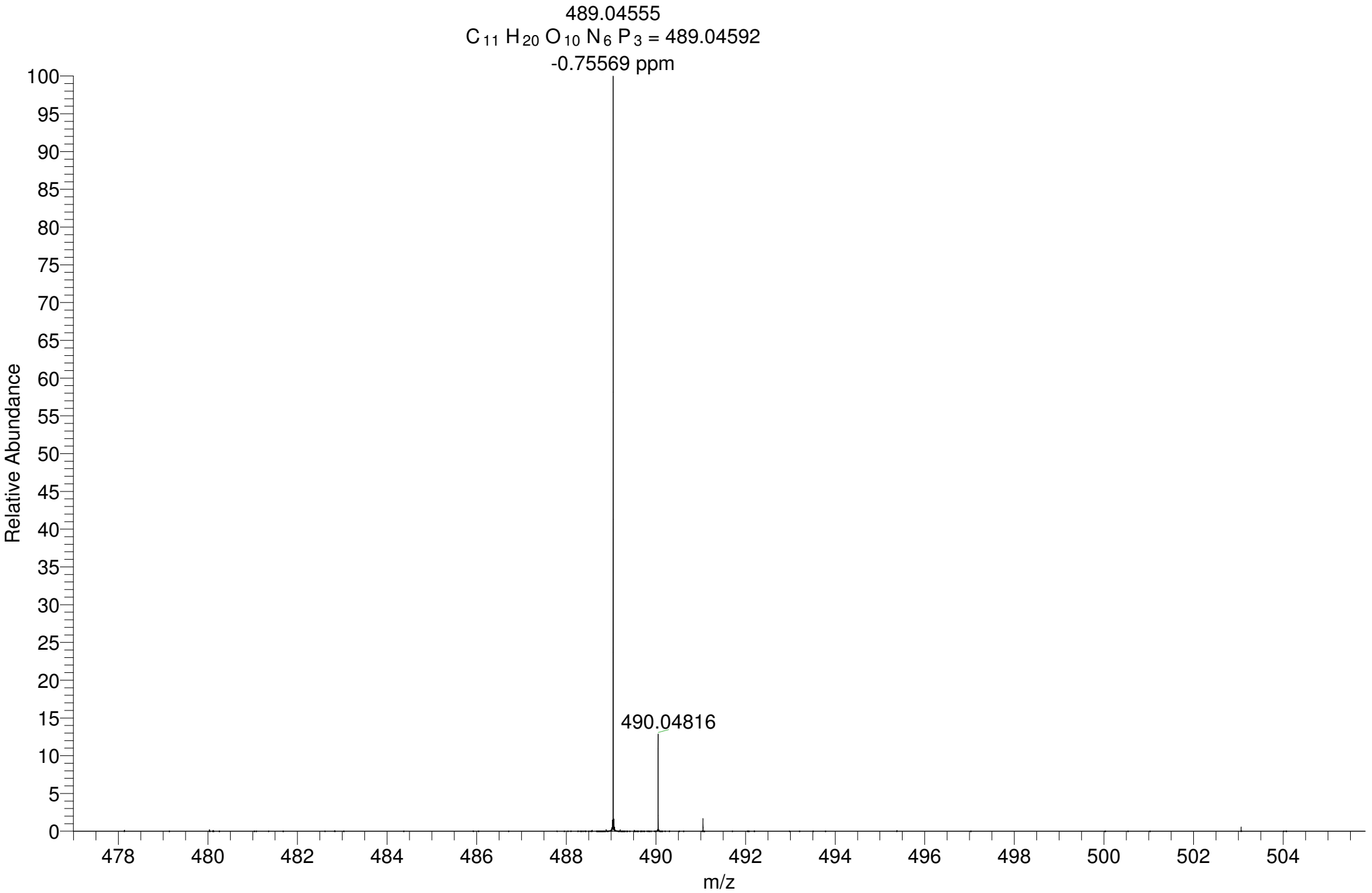
2.00
2.17
2.02
2.07
2.34
2.16
20.80
2.37
32.16

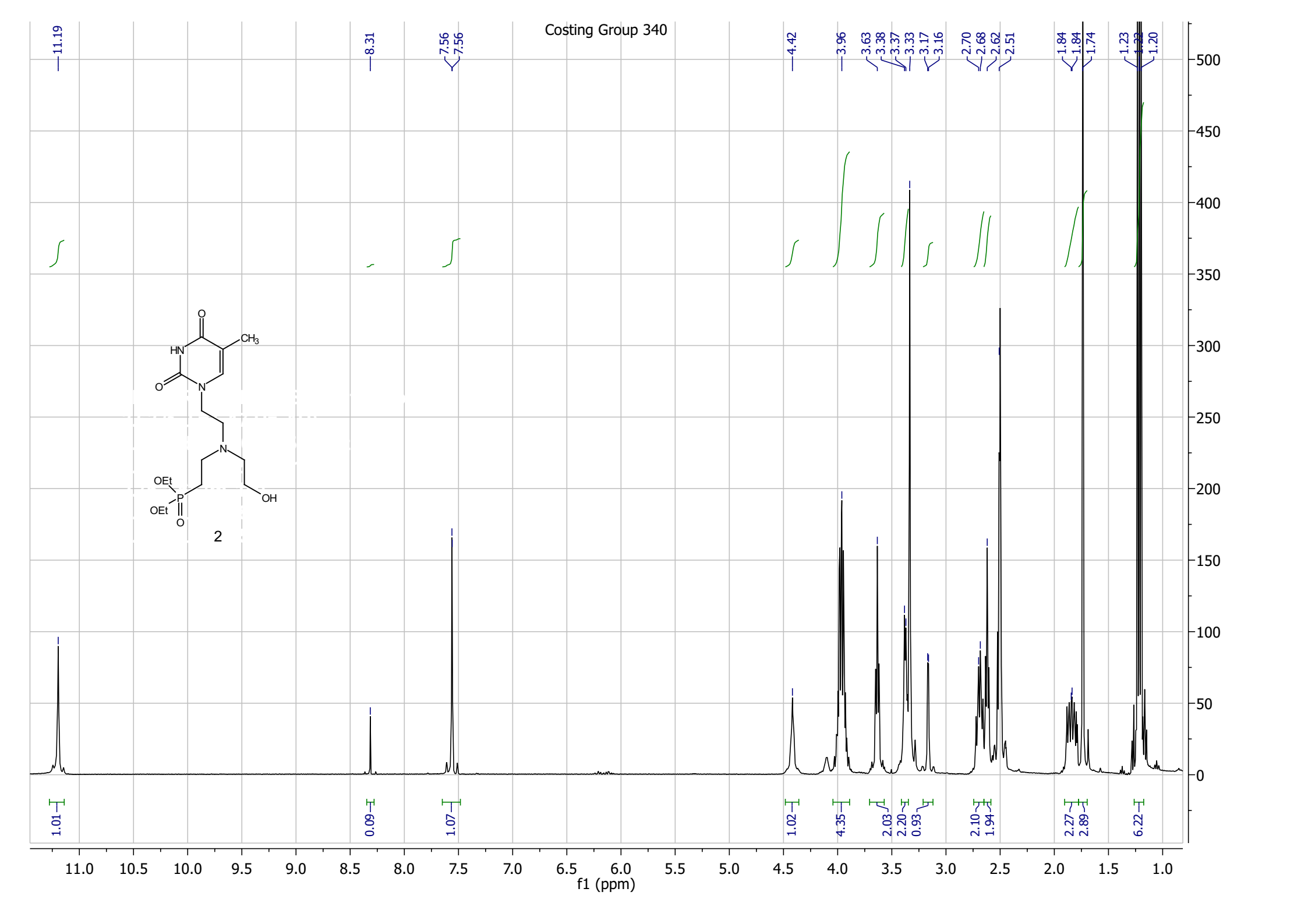
f1 (ppm)

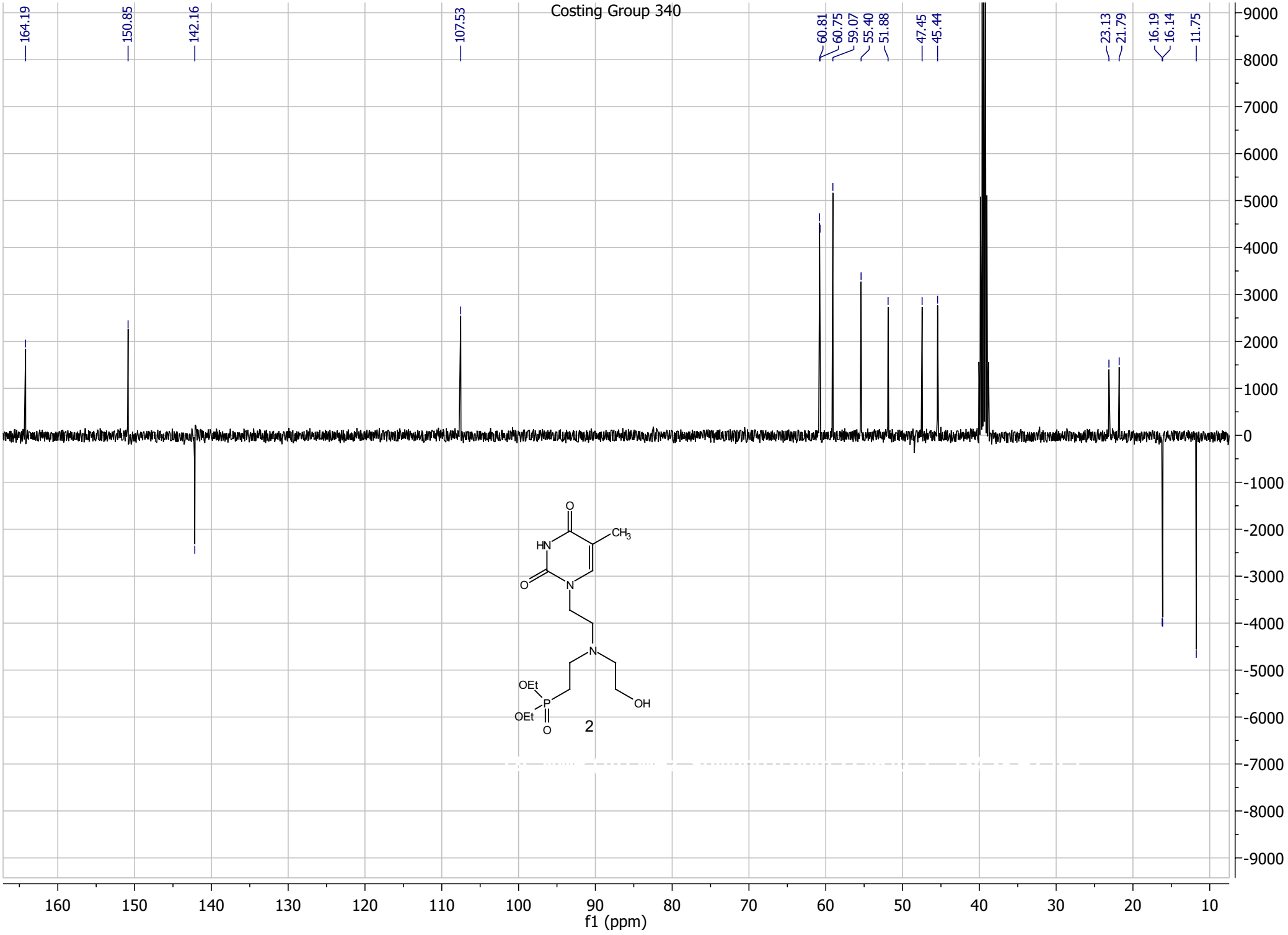

Costing Group 340
154.61
151.50
147.09
141.82
117.40
109.00
56.33
53.48
50.73
47.49
45.69
45.69
39.55
23.03
21.72
7.25
3000
2500
2000
1500
1000
500
0
-500
-1000
-1500
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)
NH2
N
OH
O
P
HO
19

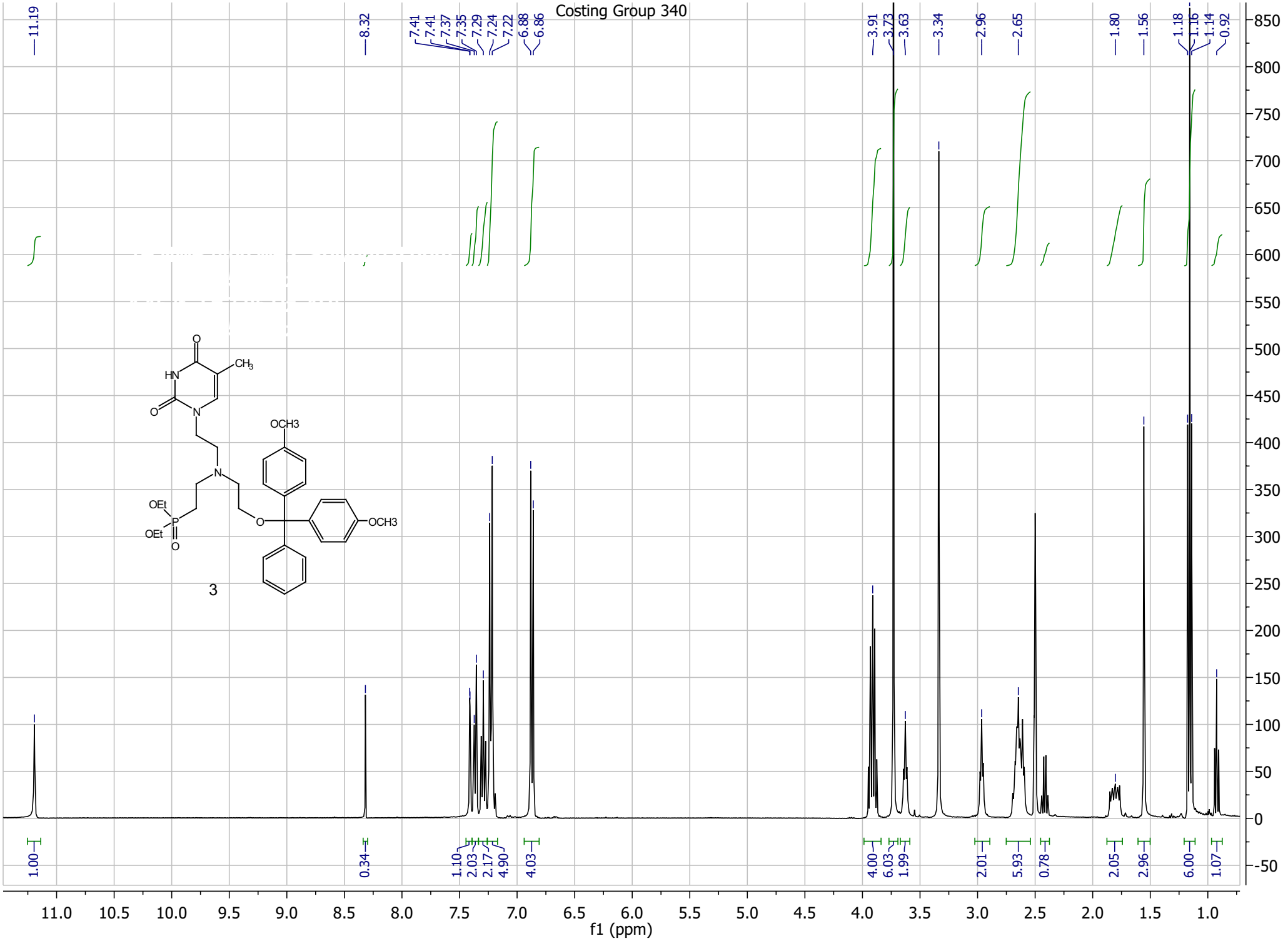

Costing Group 340
13.71
13.61
-7.63
-7.75
-22.86
-23.00
-23.14
NH2
N
N
N
N
N
OH
OH
OH
P
O
P
O
P
O
OH
HO
19
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
-100
-200
16
14
12
10
8
6
4
2
0
-2
-4
-6
-8
-10
-12
-14
-16
-18
-20
-22
-24
-26
-28
-30
f1 (ppm)

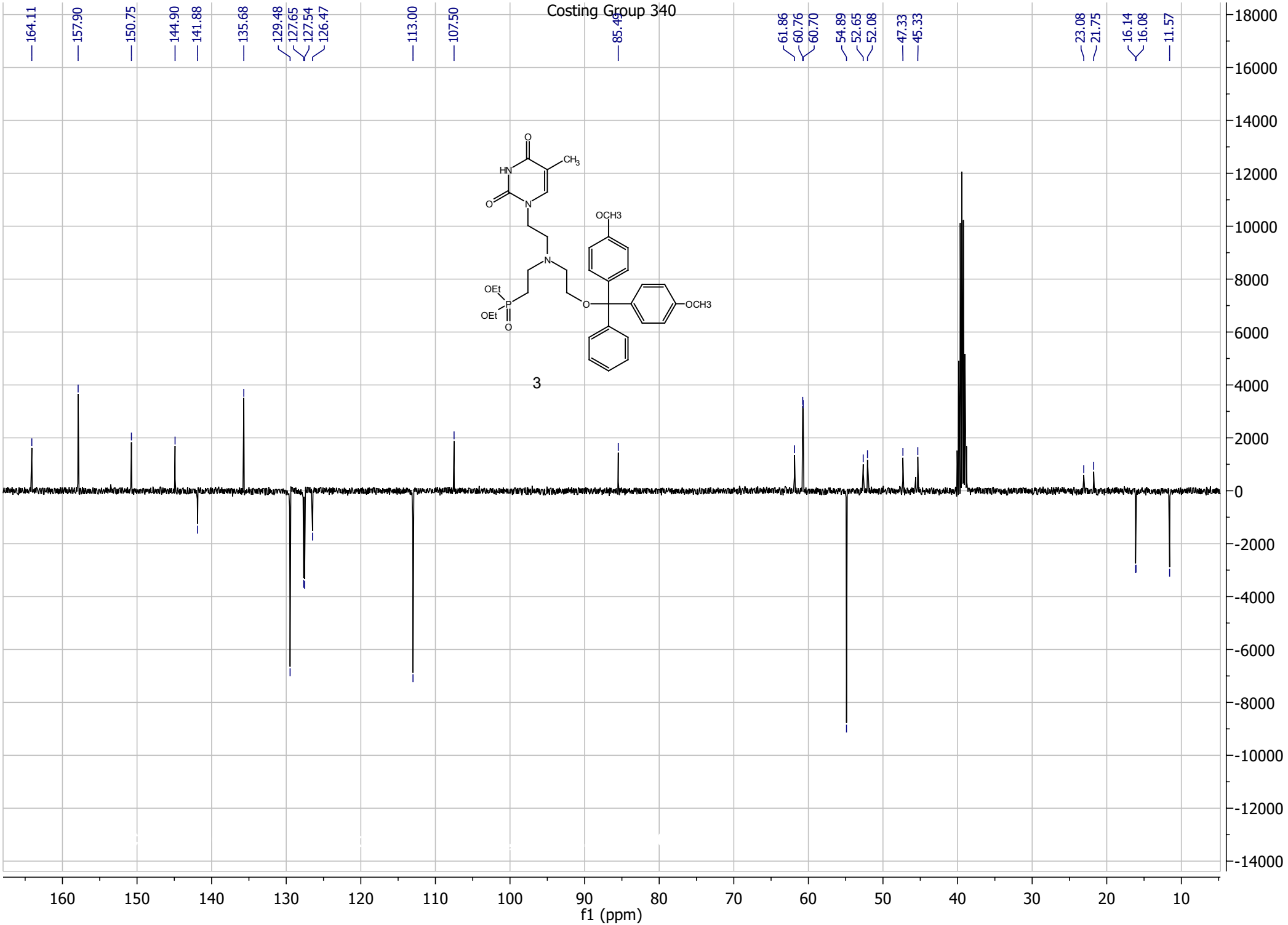
230115servisHR_13 #59-60 RT: 1.70-1.73 AV: 2 NL: 5.18E7
T: FTMS - p ESI Full ms [200.00-2000.00]

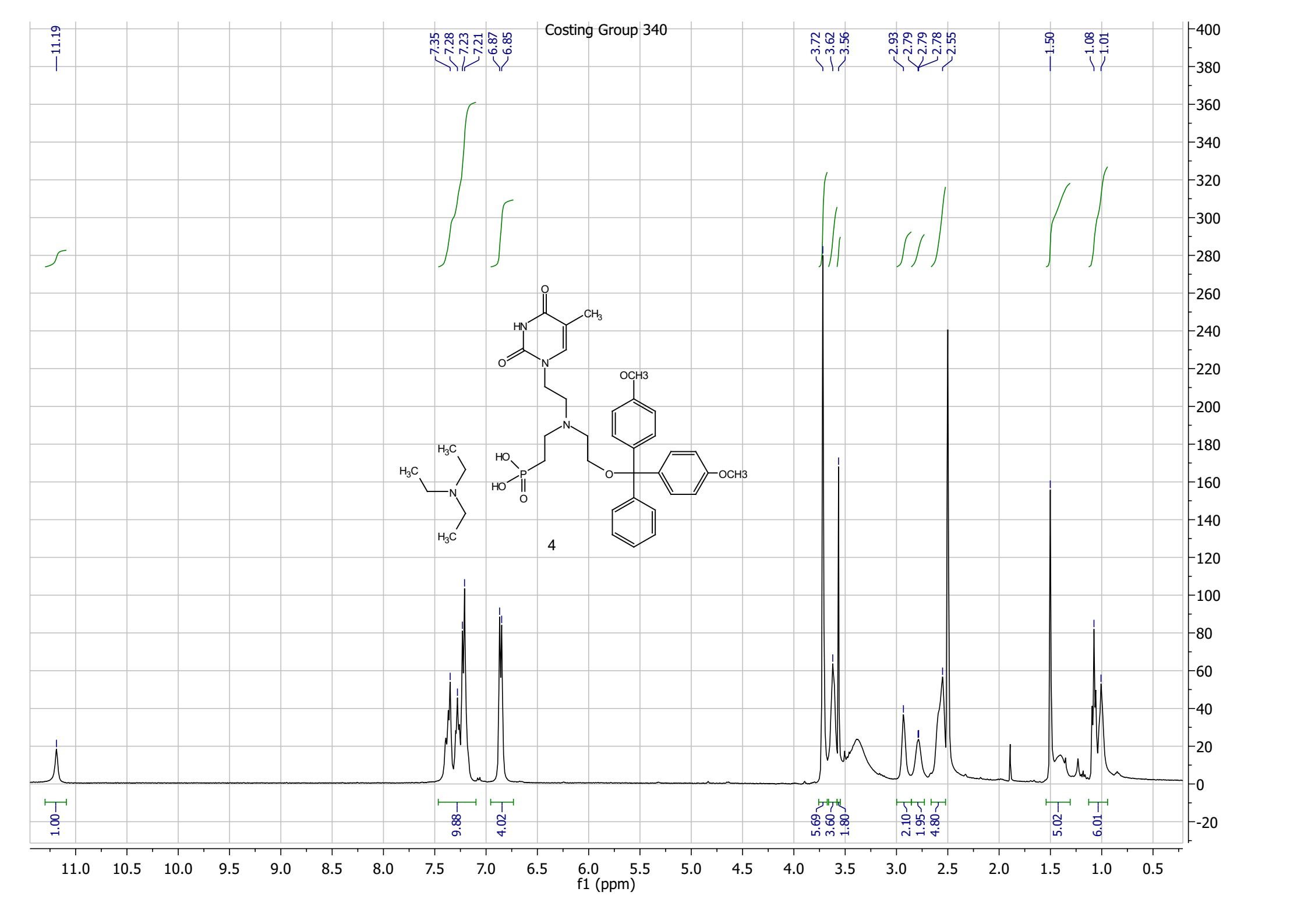


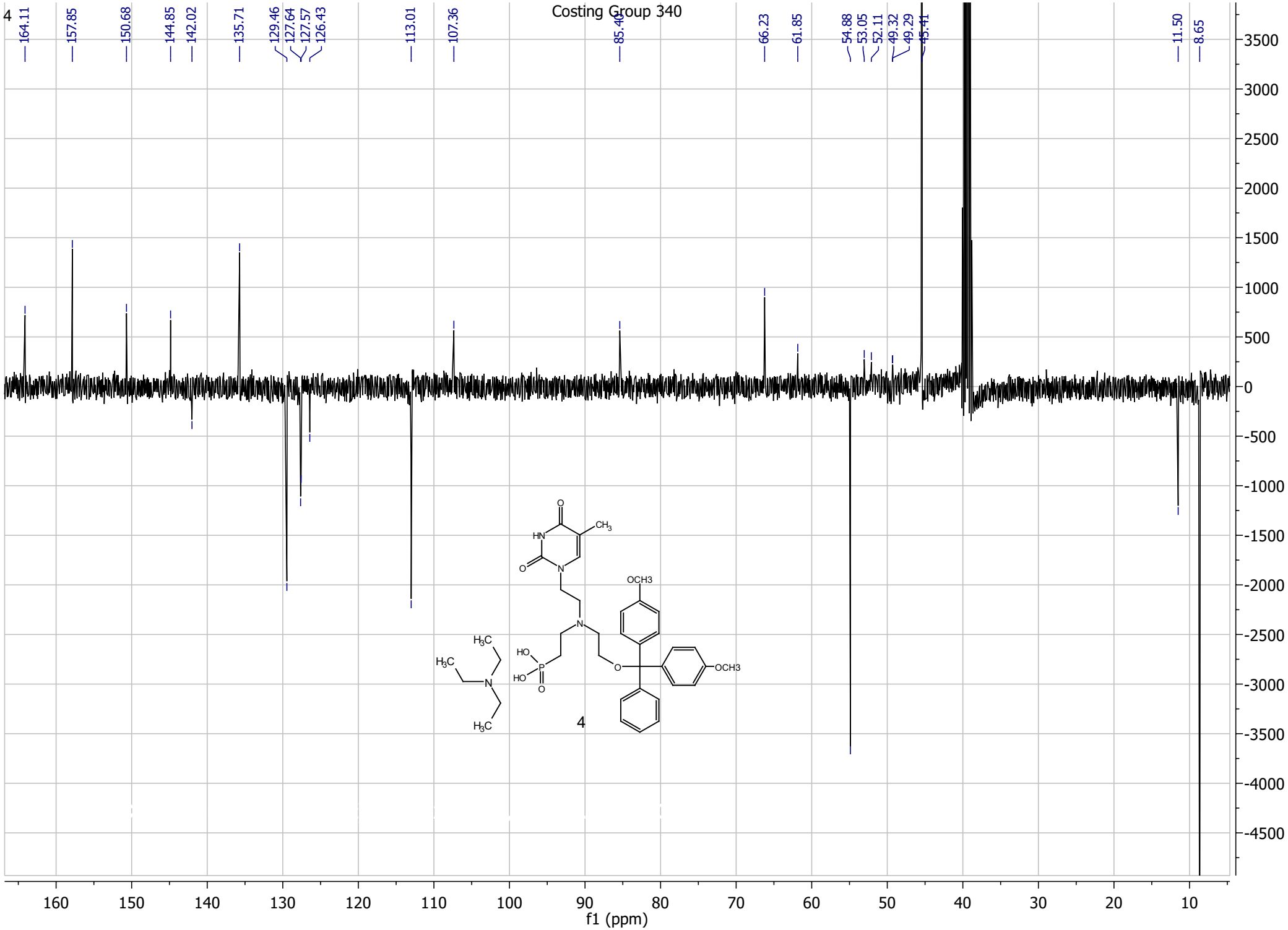

Costing Group 340
11.19
8.31
7.56
7.56
4.42
3.96
3.63
3.38
3.37
3.33
3.17
3.16
2.70
2.68
2.62
2.51
1.84
1.84
1.74
1.23
1.20
HN
O
O
N
CH3
N
OEt
OEt
P
O
OH
2
1.01
0.09
1.07
1.02
4.35
2.03
2.20
0.93
2.10
1.94
2.27
2.89
6.22
f1 (ppm)

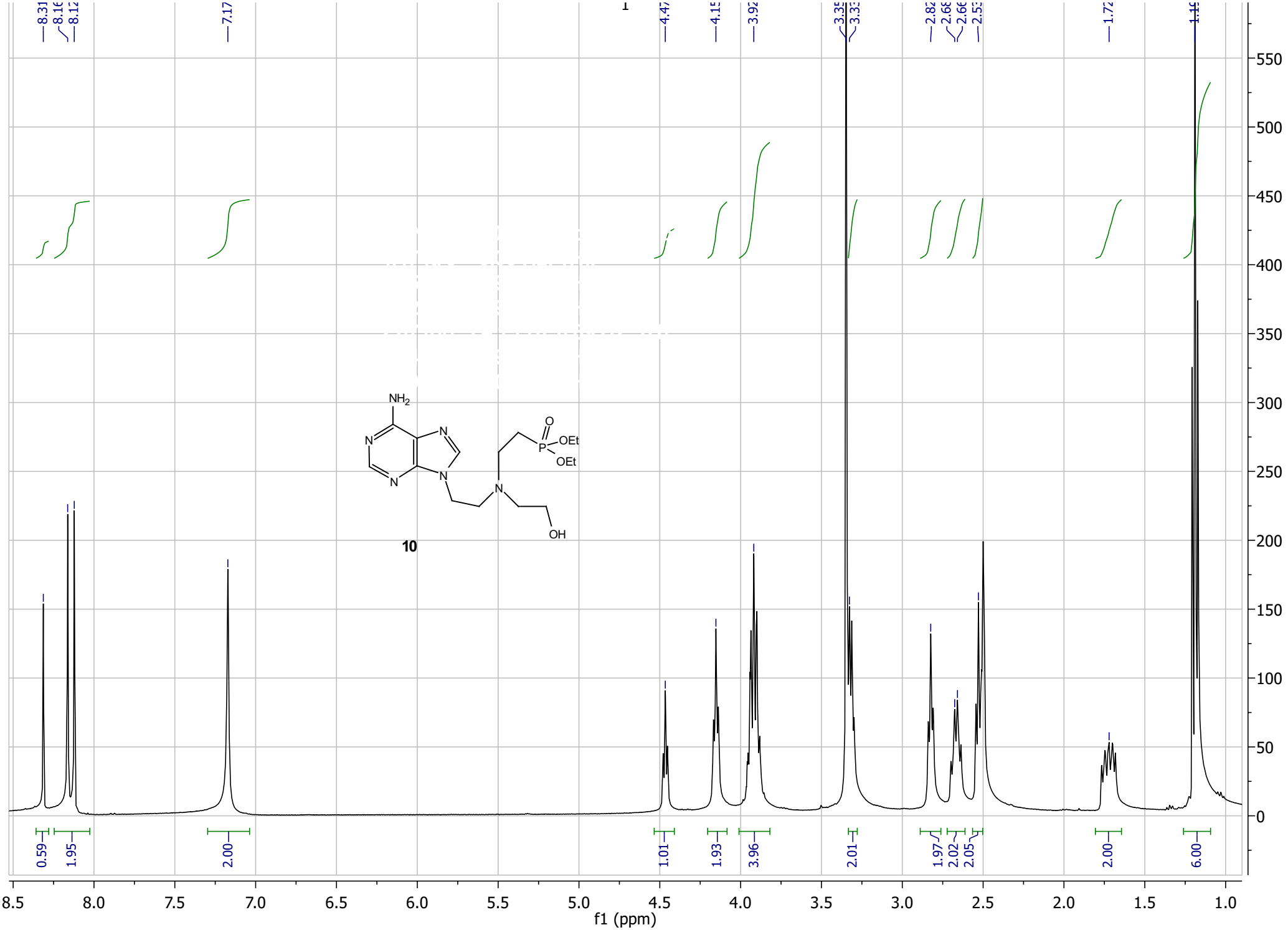

Costing Group 340
164.19
150.85
142.16
107.53
60.81
60.75
59.07
55.40
51.88
47.45
45.44
23.13
21.79
16.19
16.14
11.75
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
-2000
-3000
-4000
-5000
-6000
-7000
-8000
-9000
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)
O
HN
CH3
O
N
N
OEt
P
OEt
O
OH
2

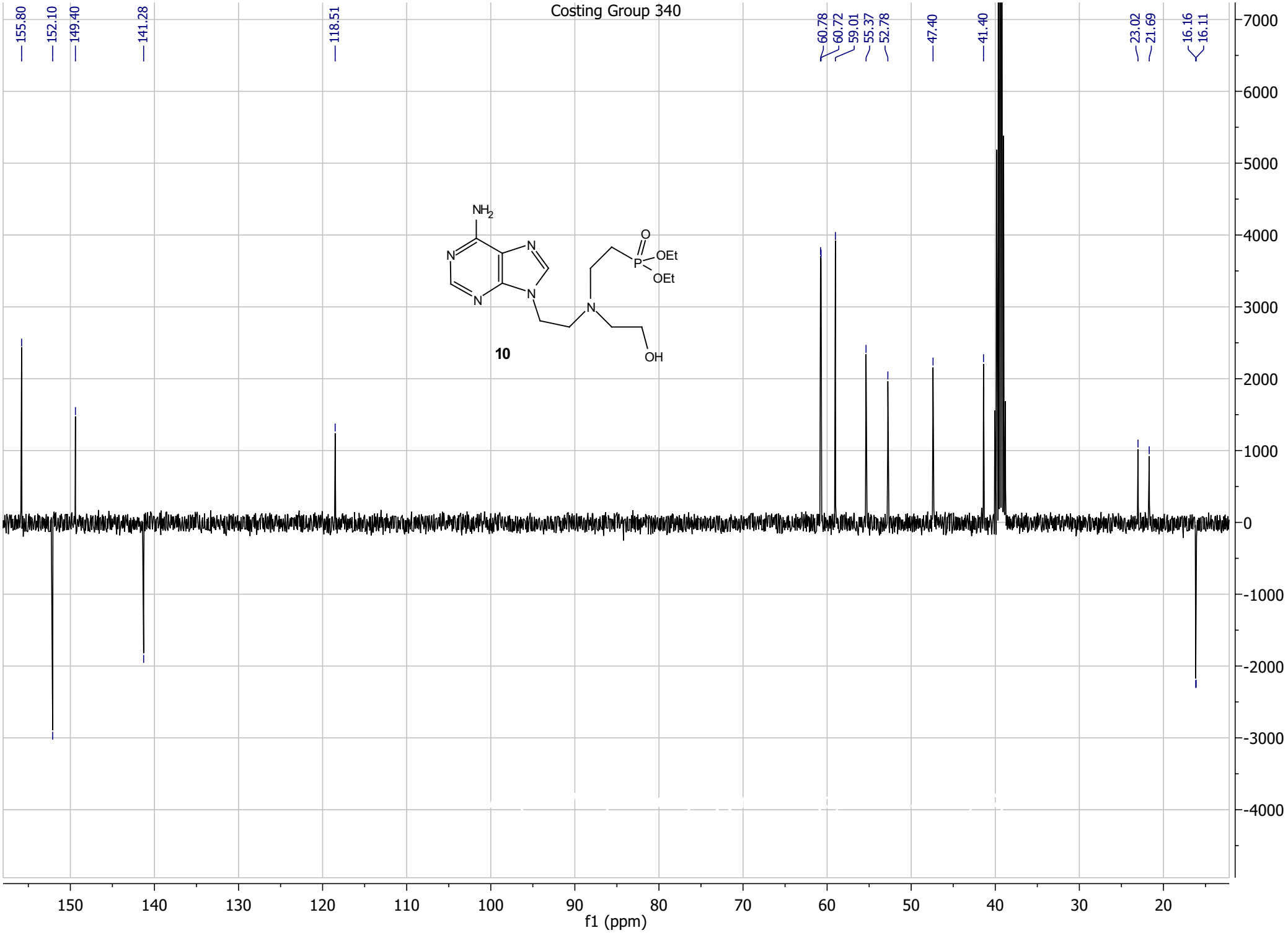

Costing Group 340
11.19
8.32
7.41
7.41
7.37
7.35
7.29
7.24
7.22
6.88
6.86
3.91
3.73
3.63
3.34
2.96
2.65
1.80
1.56
1.18
1.16
1.14
0.92
HN
O
O
N
CH3
OCH3
N
OEt
OEt
P
O
O
OCH3
3
1.00
0.34
1.10
2.03
2.17
4.90
4.03
4.00
6.03
1.99
2.01
5.93
0.78
2.05
2.96
6.00
1.07
f1 (ppm)

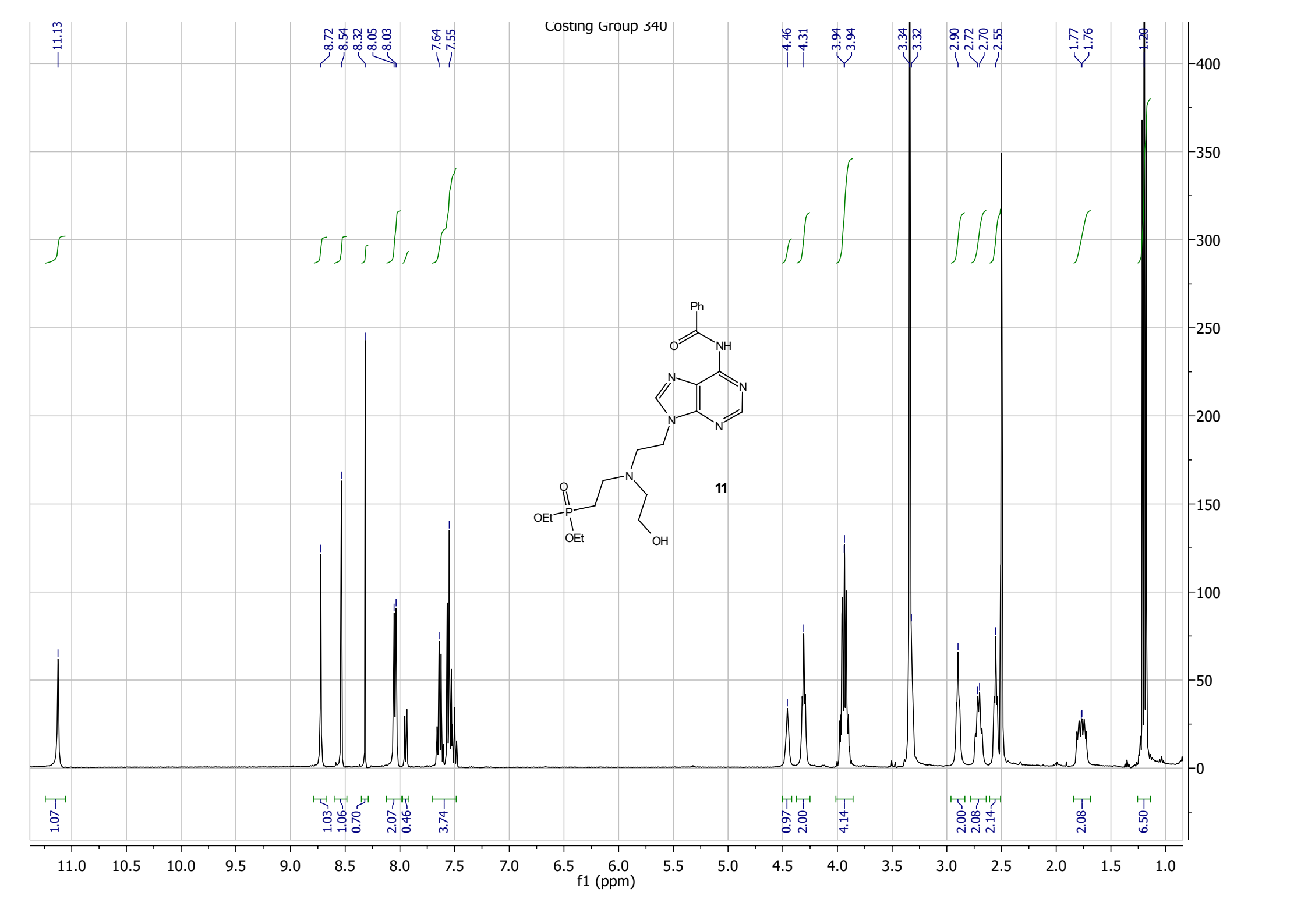

Costing Group 340
164.11
157.90
150.75
144.90
141.88
135.68
129.48
127.65
127.54
126.47
113.00
107.50
85.49
61.86
60.76
60.70
54.89
52.65
52.08
47.33
45.33
23.08
21.75
16.14
16.08
11.57
HN
O
CH3
N
OCH3
OEt
OEt
P
O
O
OCH3
3
f1 (ppm)

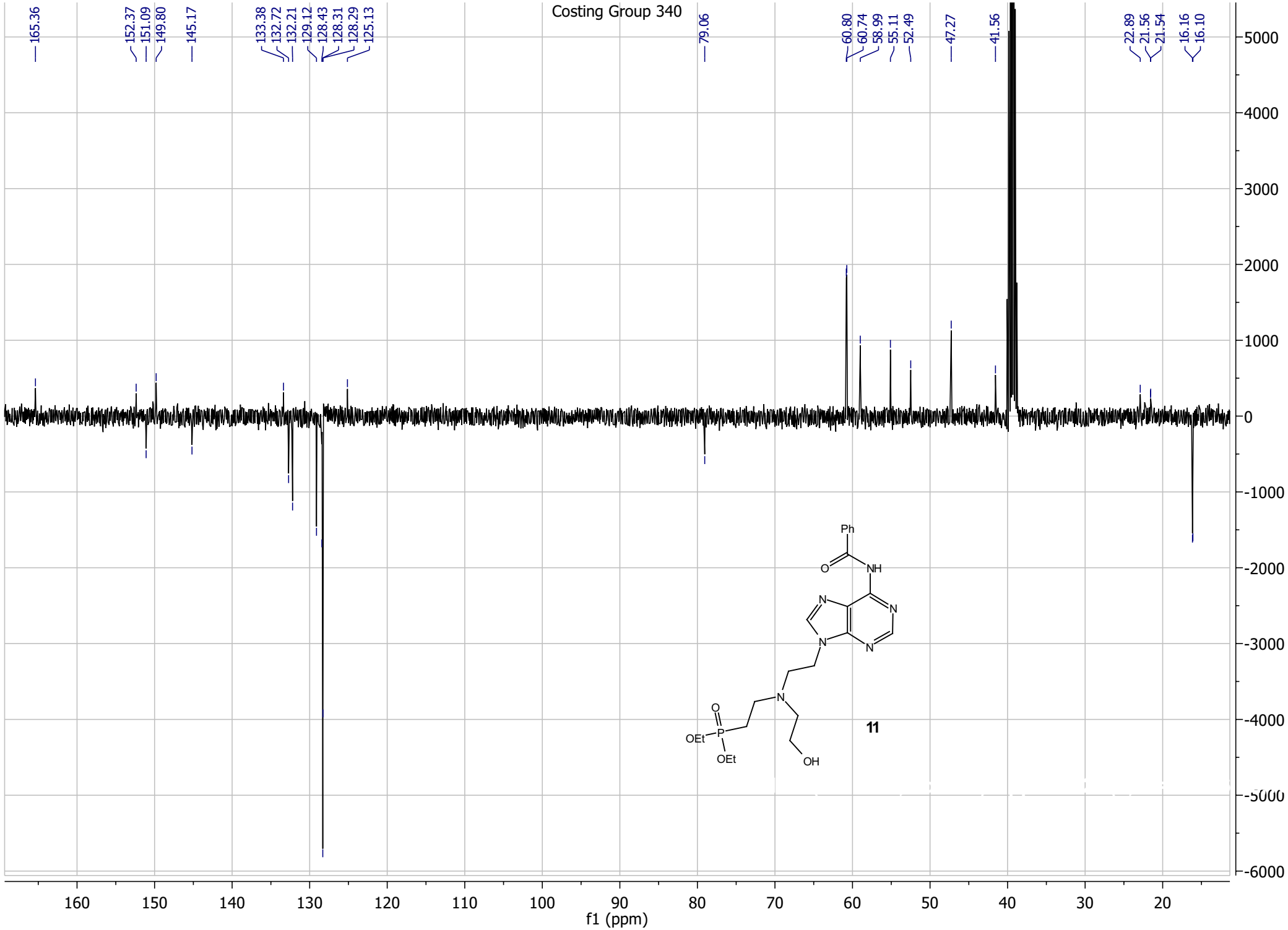

Costing Group 340
11.19
7.35
7.28
7.23
7.21
6.87
6.85
3.72
3.62
3.56
2.93
2.79
2.79
2.78
2.55
1.50
1.08
1.01
HN
O
CH3
N
OCH3
HO
P
HO
O
O
OCH3
H3C
H3C
N
H3C
4
1.00
9.88
4.02
5.69
3.60
1.80
2.10
1.95
4.80
5.02
6.01
f1 (ppm)

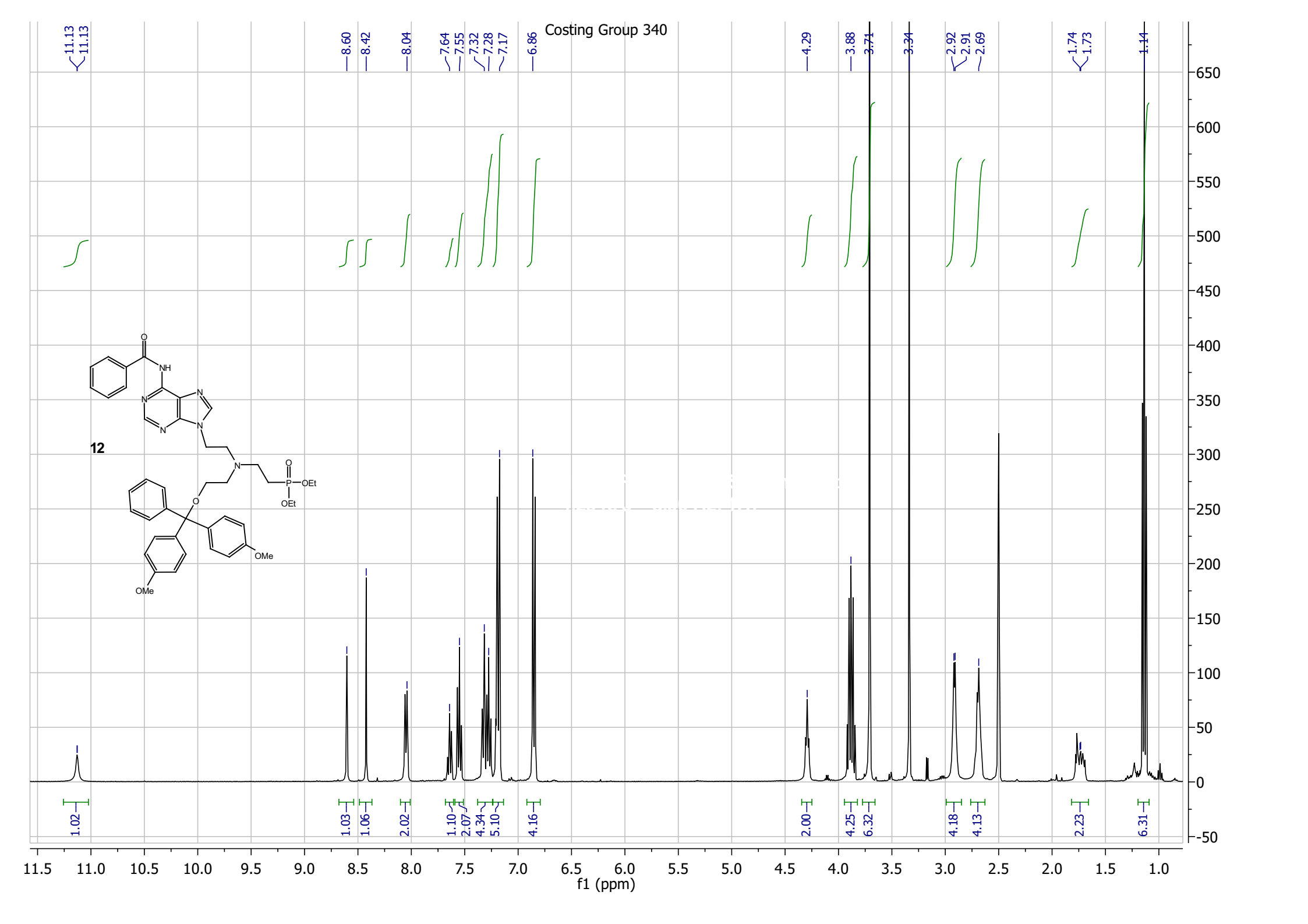

4
Costing Group 340
164.11
157.85
150.68
144.85
142.02
135.71
129.46
127.64
127.57
126.43
113.01
107.36
85.40
66.23
61.85
54.88
53.05
52.11
49.32
49.29
45.41
11.50
8.65
3500
3000
2500
2000
1500
1000
500
0
-500
-1000
-1500
-2000
-2500
-3000
-3500
-4000
-4500
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)
HN
O
CH3
N
OCH3
HO
P
O
H3C
H3C
H3C
4

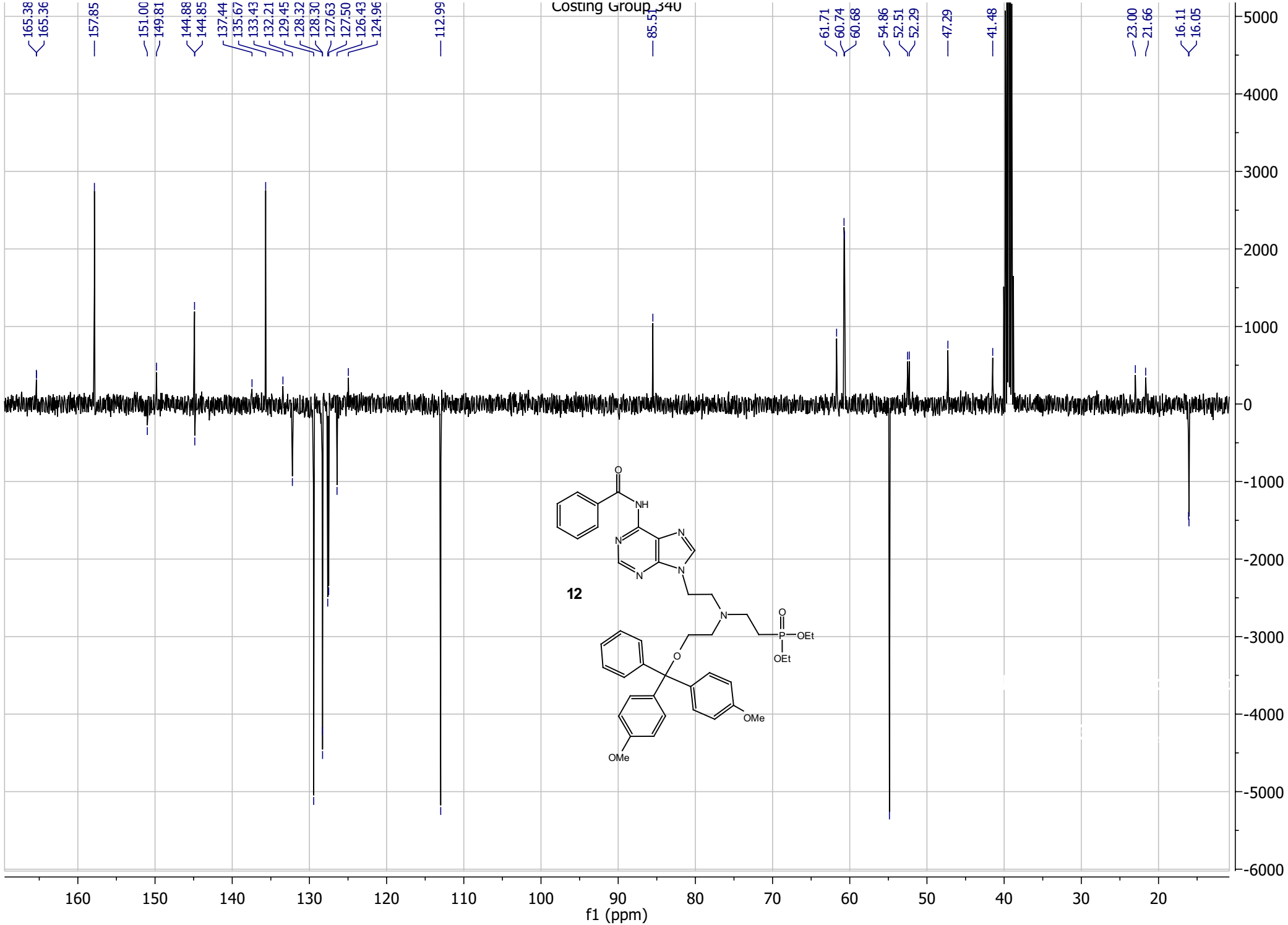

8.31
8.16
8.12
7.17
4.47
4.15
3.92
3.35
3.33
2.82
2.68
2.66
2.53
1.72
1.15
NH2
N
N
N
N
N
P
O
OEt
OEt
OH
10
0.59
1.95
2.00
1.01
1.93
3.96
2.01
1.97
2.02
2.05
2.00
6.00
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
f1 (ppm)
550
500
450
400
350
300
250
200
150
100
50
0

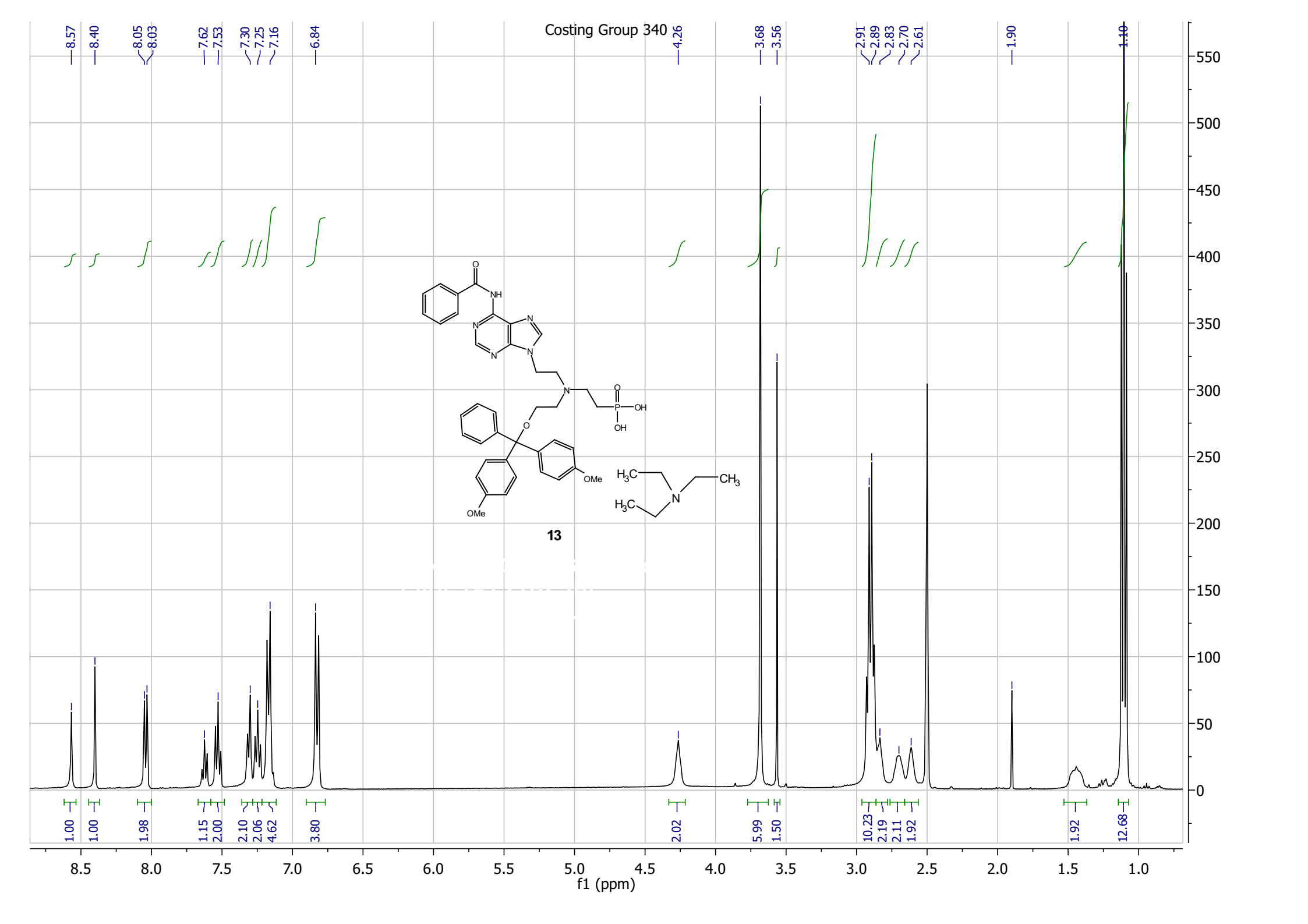

Costing Group 340
155.80
152.10
149.40
141.28
118.51
60.78
60.72
59.01
55.37
52.78
47.40
41.40
23.02
21.69
16.16
16.11
NH2
N
N
N
N
O
P
OEt
OEt
N
OH
10
7000
6000
5000
4000
3000
2000
1000
0
-1000
-2000
-3000
-4000
150
140
130
120
110
100
90
80
70
60
50
40
30
20
f1 (ppm)

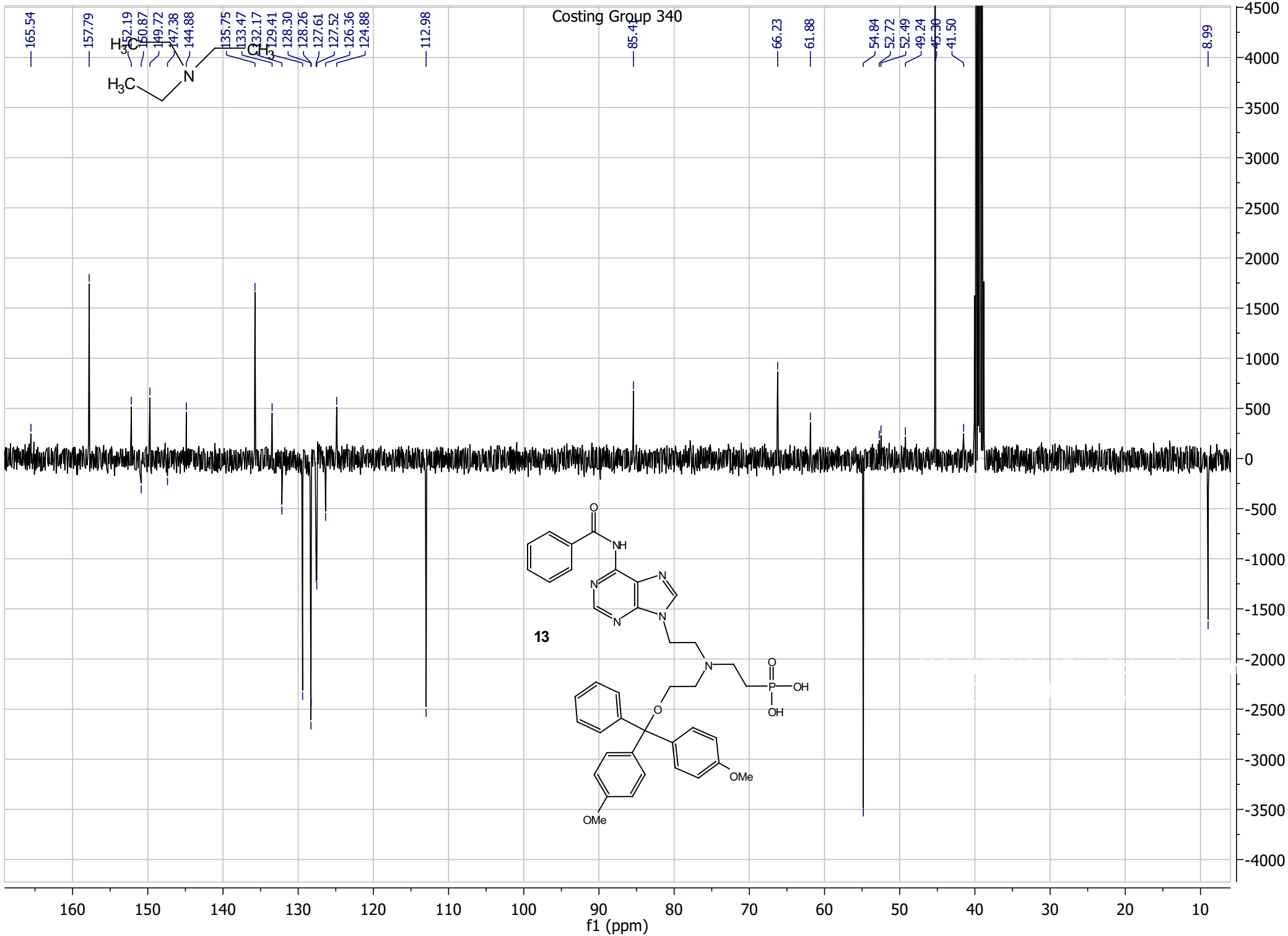

Costing Group 340
11.13
8.72
8.54
8.32
8.05
8.03
7.64
7.55
4.46
4.31
3.94
3.94
3.34
3.32
2.90
2.72
2.70
2.55
1.77
1.76
1.20
Ph
O
NH
N
N
N
N
N
O
OEt
P
OEt
OH
11
1.07
1.03
1.06
0.70
2.07
0.46
3.74
0.97
2.00
4.14
2.00
2.08
2.14
2.08
6.50
f1 (ppm)

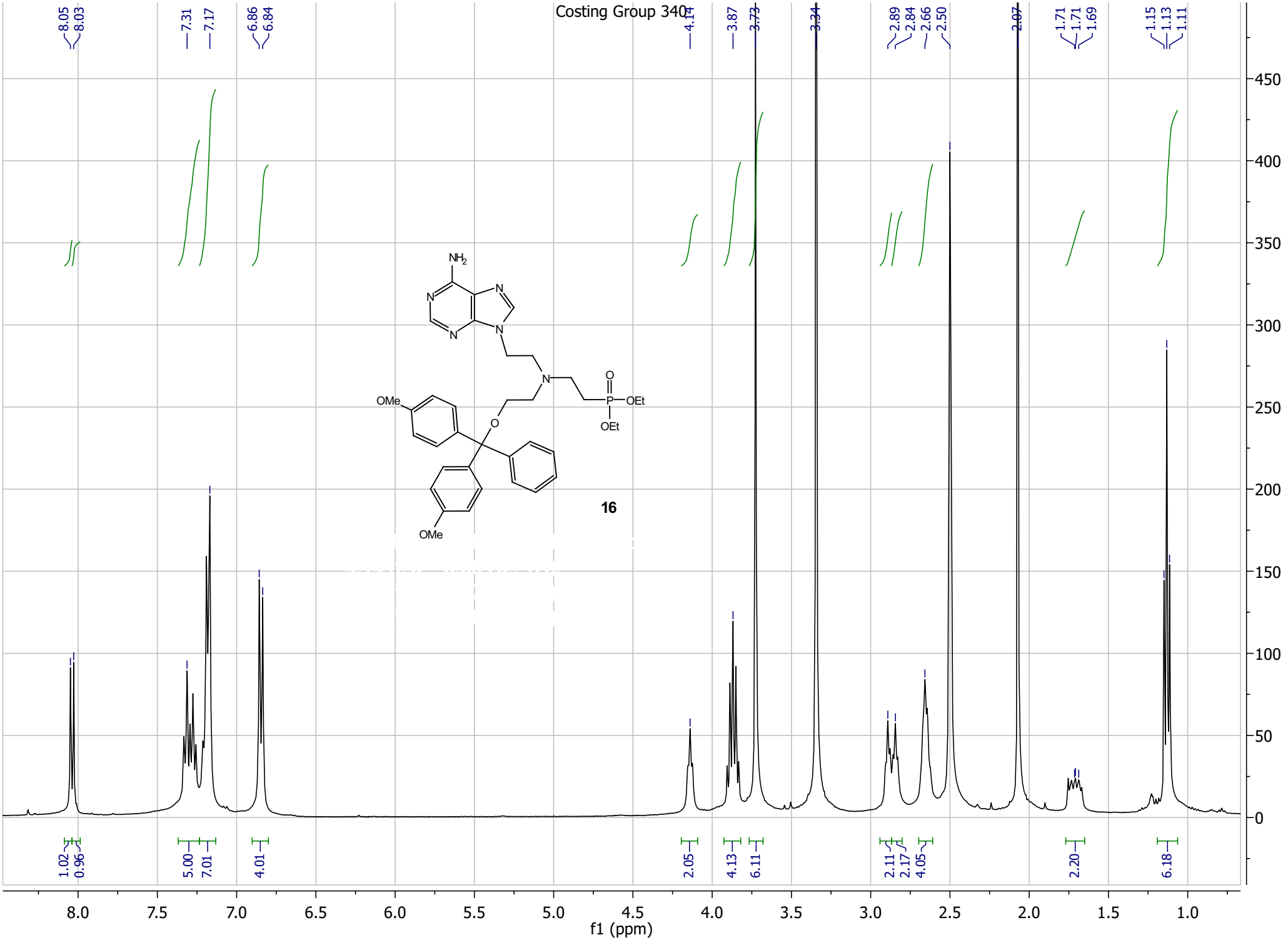

Costing Group 340
165.36
152.37
151.09
149.80
145.17
133.38
132.72
132.21
129.12
128.43
128.31
128.29
125.13
79.06
60.80
60.74
58.99
55.11
52.49
47.27
41.56
22.89
21.56
21.54
16.16
16.10
5000
4000
3000
2000
1000
0
-1000
-2000
-3000
-4000
-5000
-6000
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
f1 (ppm)
Ph
O
NH
N
N
N
N
N
O
OEt
P
OEt
OH
11


Costing Group 340
11.13
11.13
8.60
8.42
8.04
7.64
7.55
7.32
7.28
7.17
6.86
4.29
3.88
3.71
3.34
2.92
2.91
2.69
1.74
1.73
1.14
12
1.02
1.03
1.06
2.02
1.10
2.07
4.34
5.10
4.16
2.00
4.25
6.32
4.18
4.13
2.23
6.31
f1 (ppm)


Costing Group 340
165.38
165.36
157.85
151.00
149.81
144.88
144.85
137.44
135.67
133.43
132.21
129.45
128.32
128.30
127.63
127.50
126.43
124.96
112.99
85.51
61.71
60.74
60.68
54.86
52.51
52.29
47.29
41.48
23.00
21.66
16.11
16.05
12
f1 (ppm)


Costing Group 340
8.57
8.40
8.05
8.03
7.62
7.53
7.30
7.25
7.16
6.84
4.26
3.68
3.56
2.91
2.89
2.83
2.70
2.61
1.90
1.10
1.00
1.00
1.98
1.15
2.00
2.10
2.06
4.62
3.80
2.02
5.99
1.50
10.23
2.19
2.11
1.92
1.92
12.68
f1 (ppm)
OMe
OMe
OH
OH
CH3
H3C
H3C
NH
13


Costing Group 340
165.54
157.79
152.19
150.87
149.72
147.38
144.88
135.75
133.47
132.17
129.41
128.30
128.26
127.61
127.52
126.36
124.88
112.98
85.41
66.23
61.88
54.84
52.72
52.49
49.24
45.30
41.50
8.99
13
f1 (ppm)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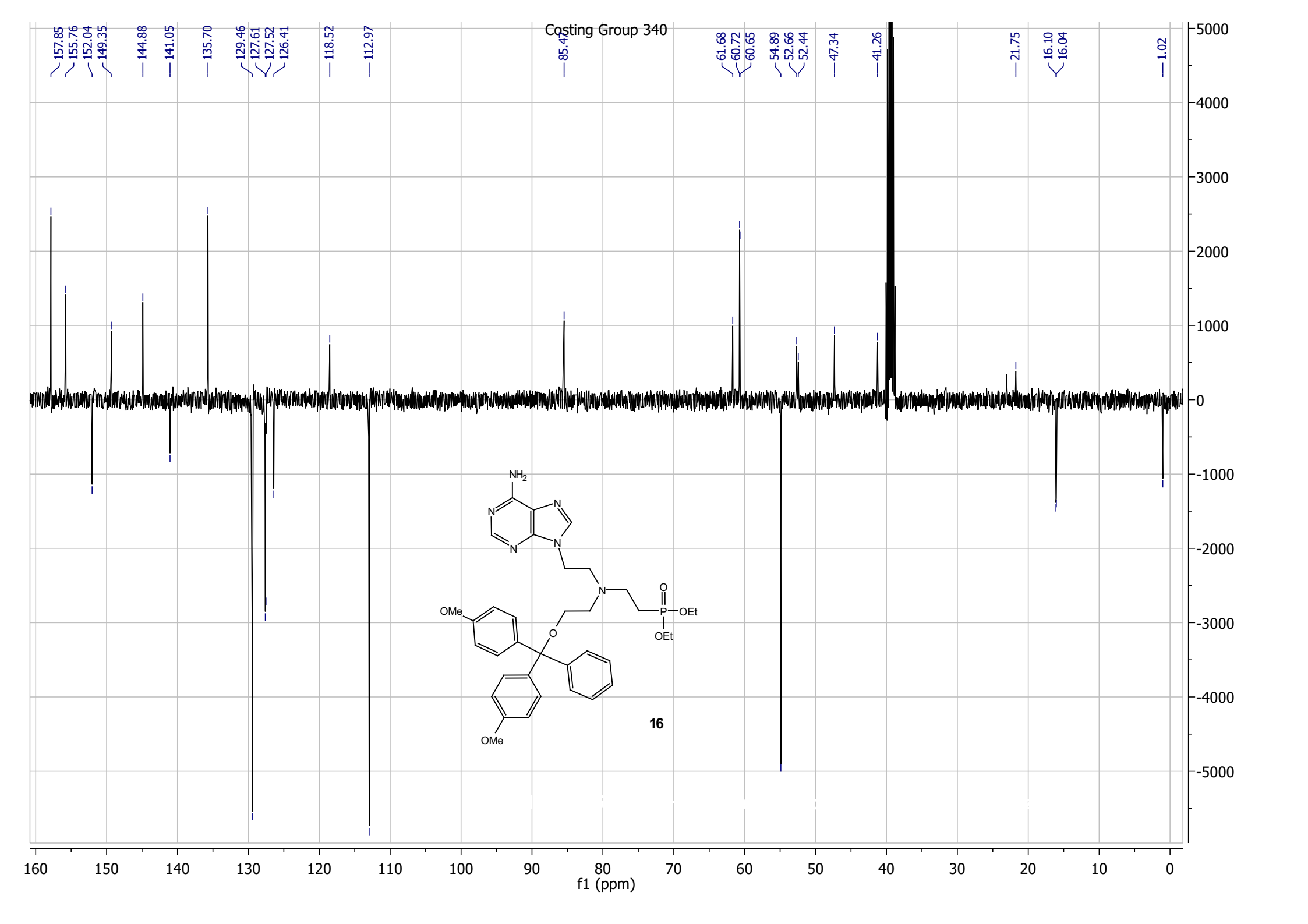
8.03
7.31
7.17
6.86
6.84
4.14
3.87
3.73
3.34
2.89
2.84
2.66
2.50
2.07
1.71
1.71
1.69
1.15
1.13
1.11
NH2
N
N
N
N
N
O
P
OEt
OEt
OMe
O
OMe
16
1.02
0.96
5.00
7.01
4.01
2.05
4.13
6.11
2.11
2.17
4.05
2.20
6.18
f1 (ppm)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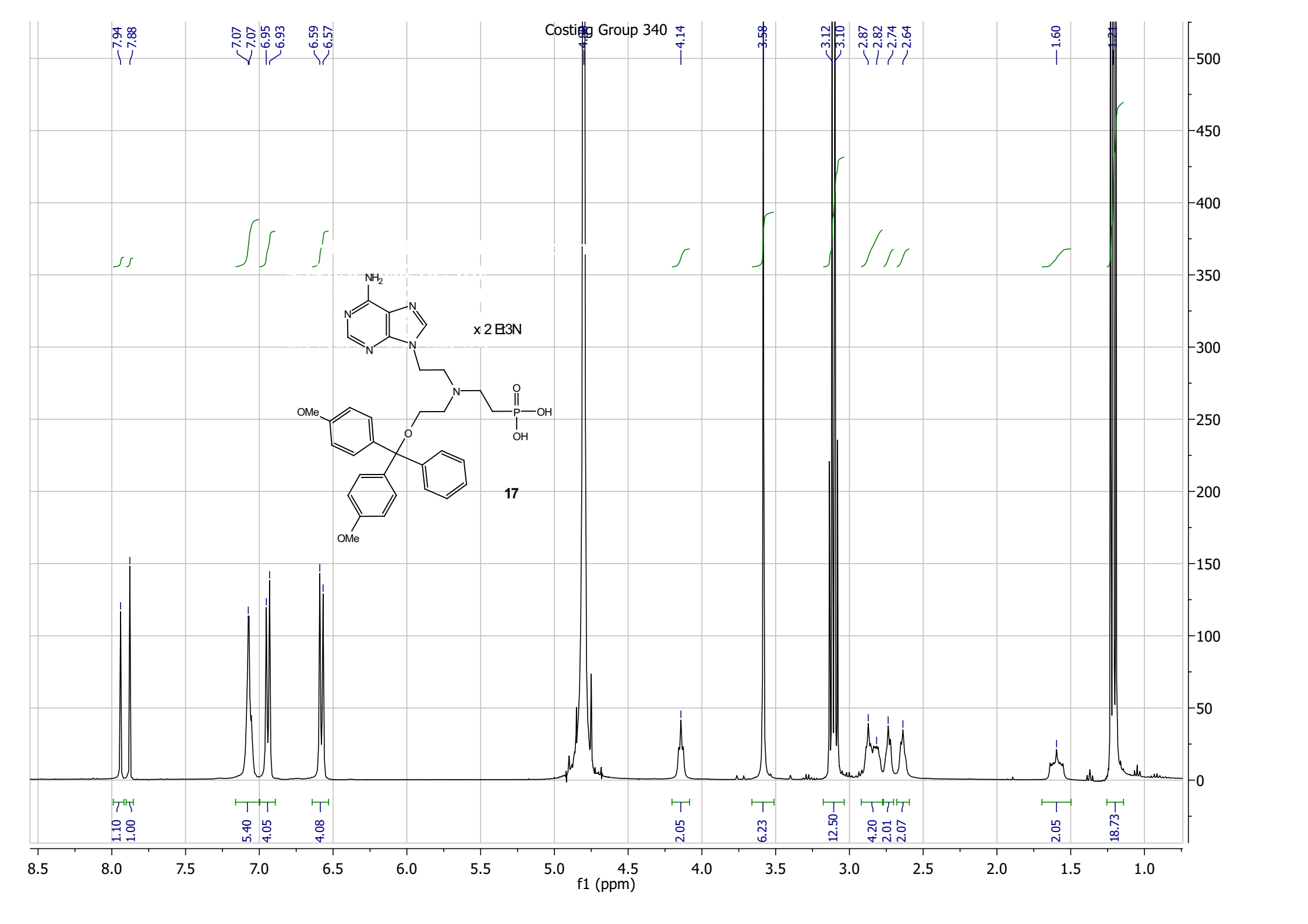
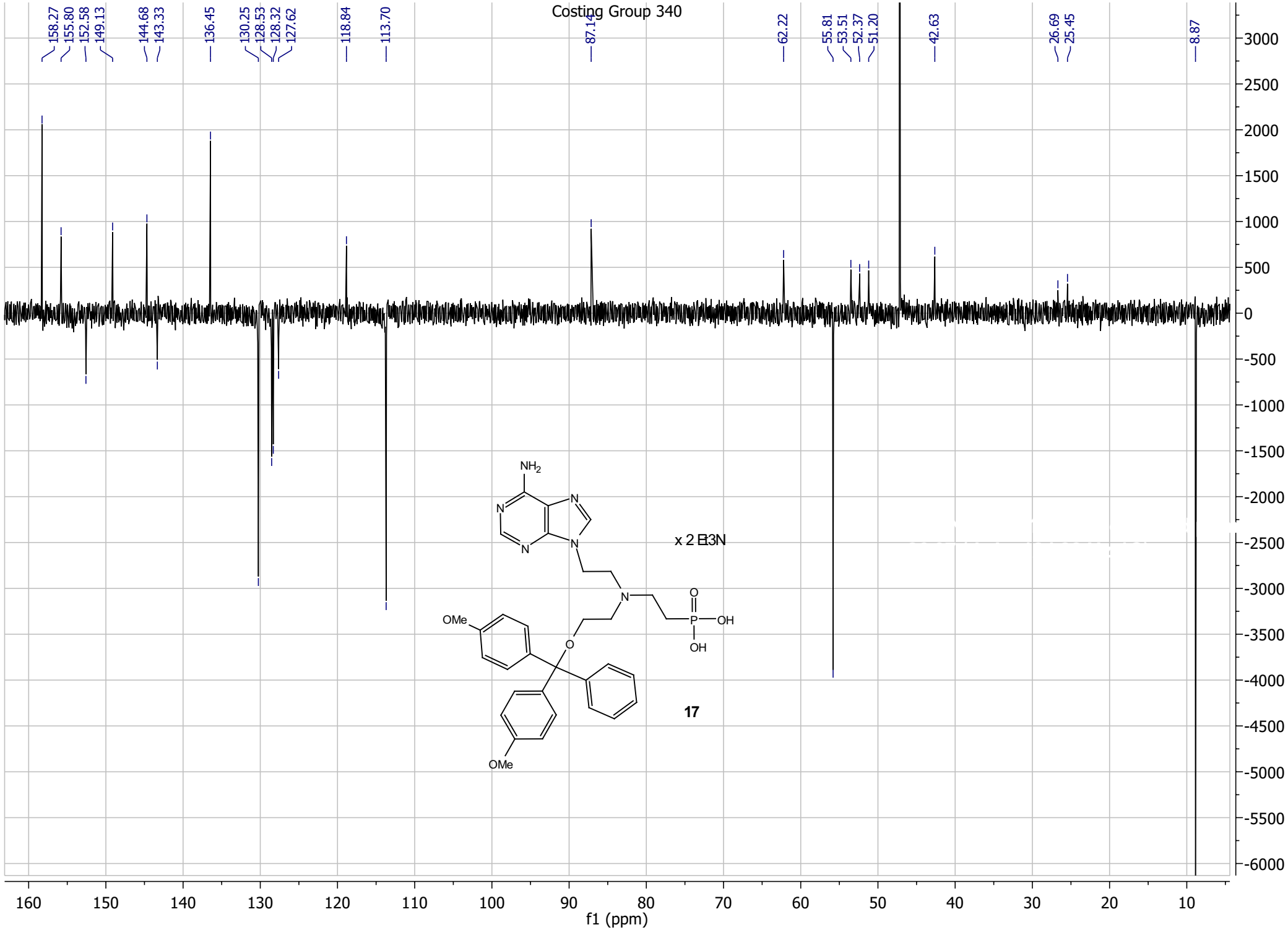
157.85
155.76
152.04
149.35
144.88
141.05
135.70
129.46
127.61
127.52
126.41
118.52
112.97
85.47
61.68
60.72
60.65
54.89
52.66
52.44
47.34
41.26
21.75
16.10
16.04
1.02
NH2
N
N
N
N
N
O
P
OEt
OEt
OMe
O
OMe
16
5000
4000
3000
2000
1000
0
-1000
-2000
-3000
-4000
-5000
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)


Costing Group 340
7.94
7.88
7.07
7.07
6.95
6.93
6.59
6.57
4.14
3.58
3.12
3.10
2.87
2.82
2.74
2.64
1.60
1.21
x 2 Et3N
17
OMe
OMe
OH
OH
1.10
1.00
5.40
4.05
4.08
2.05
6.23
12.50
4.20
2.01
2.07
2.05
18.73
f1 (ppm)


Costing Group 340
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155.80
152.58
149.13
144.68
143.33
136.45
130.25
128.53
128.32
127.62
118.84
113.70
87.14
62.22
55.81
53.51
52.37
51.20
42.63
26.69
25.45
8.87
NH2
N
N
N
N
x 2 Et3N
N
O
P
OH
OH
OMe
O
OMe
17
3000
2500
2000
1500
1000
500
0
-500
-1000
-1500
-2000
-2500
-3000
-3500
-4000
-4500
-5000
-5500
-6000
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
f1 (ppm)

Enzymatic incorporation of A-aza-ANPpp (19**) and T-aza-ANPpp (**8**) by DNA polymerases**

The enzymatic incorporation of adenosine derivative **A-aza-ANPpp** and thymidine derivative **T-aza-ANPpp** was tested using five different bacterial DNA polymerases (KOD XL, Vent(exo-), Pwo, DeepVent(exo-), Bst large fragment) and a human DNA polymearse α, on a 31-base-long template (**Temp^{Prb4basII}**), allowing the incorporation of four modifications. The modified **N-aza-ANPpp** was used instead of its natural counterpart in combination with the three remaining natural dNTPs. In all the cases, the **N-aza-ANPpp**s were not incorporated and the obtained oligonucleotides were the same as in negative controls (**Fig. 5**).

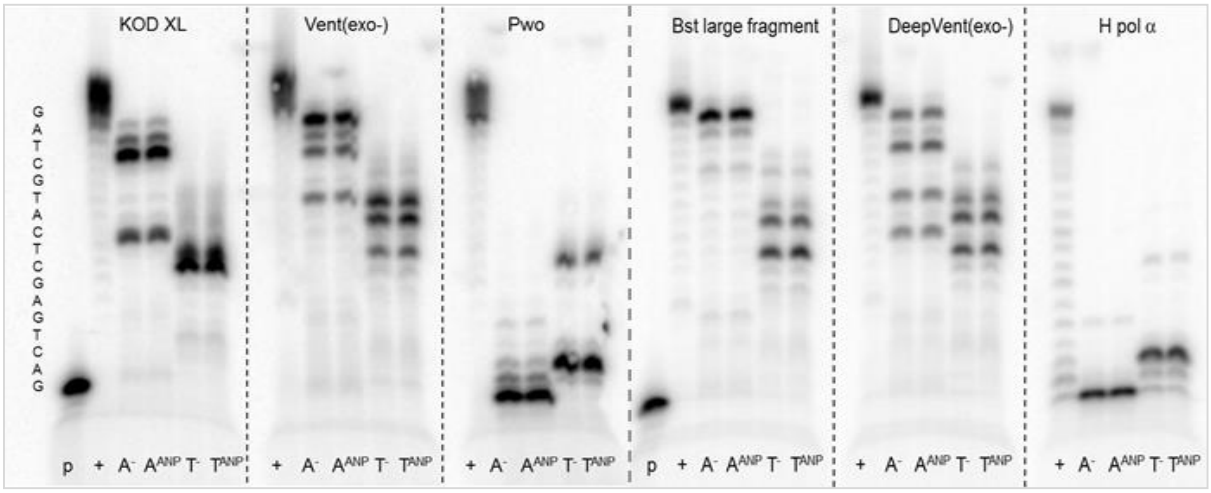

Fig. 5. Denaturing PAGE analysis of PEX products with template **Temp^{Prb4basII}**, **A-aza-ANPpp** or **T-aza-ANPpp** and KOD XL, Vent(exo-), Pwo, Bst large fragment, DeepVent(exo-) and H pol α DNA polymerases. The experiments are supplemented with a ^{32}P-radiolabelled primer (p), positive control (+: all natural dNTPs), and negative control (A^-: absence of dATP; T^-: absence of dTTP).

Inhibition of DNA polymerase activity [1] by A-aza-ANPpp (19**) and T-aza-ANPpp (**8**)**

To determine the effect of **A-aza-ANPpp** and **T-aza-ANPpp** on DNA polymerase activity, the primer was extended in the presence of all four natural dNTPs (100 μM) and various concentrations of **A-aza-ANPpp** or **T-aza-ANPpp** (100–900 μM). The gel (**Fig. 6**) demonstrates that neither bacterial polymerase KOD XL nor human polymerase α are inhibited in the presence of **A-aza-ANPpp** or **T-aza-ANPpp**.

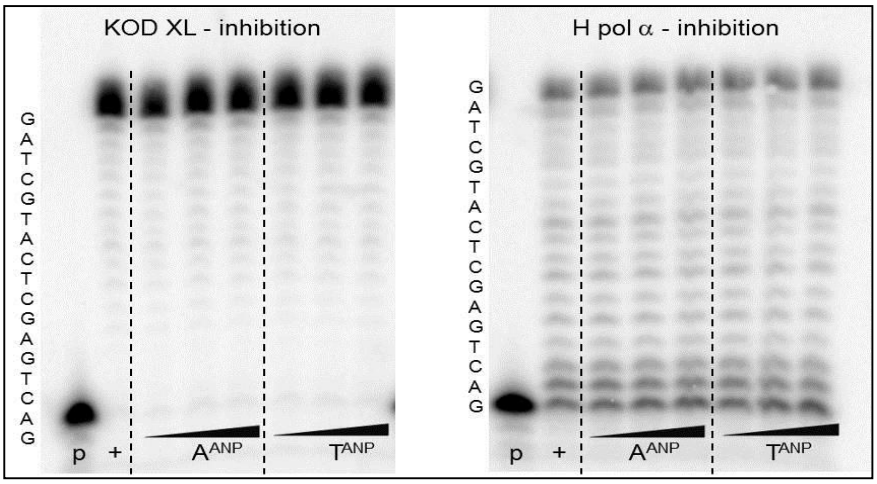

Fig. 6. Inhibition of human polymerase alpha activity by **A-aza-ANPpp** or **T-aza-ANPpp**. The reaction mixtures contained all four natural dNTPs (100 μM) together with the modified nucleoside triphosphate **A-aza-ANPpp** or **T-aza-ANPpp** (100, 300 or 900 μM). The experiments are supplemented with a ^{32}P-radiolabeled primer (p), and a positive control (+: all natural dNTPs).

Experimental:

Primer extension experiments with bacterial DNA polymerases

The reaction mixture for PEX (10 μL) contained DNA polymerase (Bst large fragment (8 U/μL, 0.5 μL), Deep Vent(exo-) (2 U/μL, 0.5 μL), Vent(exo-) (2 U/μL, 0.5 μL), KOD XL (2.5 U/μL, 0.5 μL), or Pwo (1 U/μL, 0.5 μL)), natural dNTPs (4 mM, 0.5 μL), **A-aza-ANPpp** or **T-aza-ANPpp** (4 mM, 0.5 μL), 5'-^{32}P-labelled primer (3 μM, 0.5 μL, **Prim^{248short}**: 3'-GGGTACGGCGGGTAC-5'), and 31-mer template (3 μM, 0.75 μL, **Temp^{Prb4basII}**: 5'-CTAGCATGAGCTCAGTCCCATGCCGCCCATG-3') in the buffer (1 μL) supplied by the manufacturer with the enzyme (ThermoPol buffer for Bst large fragment, DeepVent(exo-) and Vent(exo-), KOD XL buffer, or Pwo buffer). The primer was labeled using [γ^{32}P]-ATP according to standard techniques.[2]

The reaction mixtures were incubated at 60 °C for 30 min. The reactions were stopped by the addition of PAGE stop solution (20 μL, 80% [v/v] formamide, 20mM EDTA, 0.025% [w/v] bromophenol blue, 0.025% [w/v] xylene cyanol) and heated at 95 °C for 5 min. Aliquots (4 μL) were subjected to vertical electrophoresis in 12.5% denaturing polyacrylamide gel containing 1x TBE buffer (pH 8) and 7M urea at 45 mA for 50 min. The gels were dried (85 °C, 70 min), audioradiographed and visualized by phosphorimager (Typhoon 94^{10}, Amersham Biosciences).

Primer annealing

The radiolabeled primer (**Prim^{248short}**: 3'-GGGTACGGCGGGTAC-5') was mixed with the template (**Temp^{Prb4basII}**: 5'-CTAGCATGAGCTCAGACCCATGCCGCCCATG-3', 1.5 excess) in aqueous Tris-HCl (pH 7.5, 50 mM), DTT (5 mM), $MgCl_2$ (5 mM), so that the final primer concentration was 1.1 µM. The annealing was performed in a thermal cycler. The sample was first heated at 95 °C for 5 min and then slowly cooled to 25 °C over 60 min. Thus prepared primed template was stored at -20 °C.

Primer extension experiments using human DNA polymerase alpha

The reaction mixture (10 µL) contained primed template (0.1 µM primer, 0.15 µM template), human DNA polymerase alpha (4 U/µL, 0.125 µL), BSA (0.1 mg/mL), glycerol (10 %), Tris-HCl (pH 7.5, 50 mM), DTT (5 mM), $MgCl_2$ (5 mM), natural dNTPs (100 µM) and **A-aza-ANPpp** or **T-aza-ANPpp** (200 µM). The reactions were carried out at 37 °C for 90 min and were stopped by the addition of 20 µL PAGE stop solution (80% [v/v] formamide, 20mM EDTA, 0.025% [w/v] bromophenol blue, 0.025% [w/v] xylene cyanol) and heated at 95 °C for 5 min. Aliquots (4 µL) were subjected to vertical electrophoresis in 12.5% denaturing polyacrylamide gel containing 1x TBE buffer (pH 8) and 7M urea at 45 mA for 50 min. The gels were dried (85 °C, 70 min), audioradiographed and visualized by phosphorimager (Typhoon 9410, Amersham Biosciences).

Inhibition of KOD XL polymerase activity by A-aza-ANPpp and T-aza-ANPpp

The reaction mixture (10 µL) contained 5'-^{32}P-labelled primer (**Prim^{248short}**, 3 µM, 0.5 µL), template (**Temp^{Prb4basII}**, 3 µM, 0.75 µL), KOD XL DNA polymerase (2.5 U/µL, 0.5 µL, all four natural dNTPs (100 µM) and the **N-aza-ANPpp** (**A-aza-ANPpp** or **T-aza-ANPpp**) at the given concentration (100-900 µM). The reactions were carried out at 60 °C for 15 min and were stopped by adding 20 µL PAGE stop solution and heating at 95 °C for 5 min. Aliquots (4 µL) were subjected to electrophoresis in 12.5% denaturing polyacrylamide gel.

Inhibition of DNA polymerase activity by A-aza-ANPpp and T-aza-ANPpp

The reaction mixture (10 µL) contained primed template (0.1 µM primer, 0.15 µM template), human DNA polymerase alpha (4 U/µL, 0.5 U), BSA (0.1 mg/ml), glycerol (10 %), Tris-HCl (pH 7.5, 50 mM), DTT (5 mM), $MgCl_2$ (5 mM), all four natural dNTPs (100 µM) and the **N-aza-ANPpp** (**A-aza-ANPpp** or **T-aza-ANPpp**) at the given concentration (100-900 µM). The reactions were carried out at 37 °C for 60 min and were stopped by adding 20 µL PAGE stop

solution and heating at 95 °C for 5 min. Aliquots (4 μL) were subjected to electrophoresis in 12.5% denaturing polyacrylamide gel.

References:

1. Perlíková, P.; Eberlin, L.; Ménová, P.; Raindlová, V.; Slavětínská, L.; Tloušťová, E.; Bahador, G.; Lee, Y.-J.; Hocek, M. Synthesis and Cytostatic and Antiviral Activities of 2'-Deoxy-2 ',2 '-difluororibo- and 2 '-Deoxy-2 '-fluororibonucleosides Derived from 7-(Het)aryl-7-deazaadenines. *ChemMedChem* **2013**, *8*, 832–846.

2. Macíčková-Cahová, H.; Vrábel, M.; Hocek, M. Cross-Coupling Modification of Nucleoside Triphosphates, PEX, and PCR Construction of Base-Modified DNA. *Curr. Protoc. Chem. Biol.* **2010**, *2*, 1–14.