Supplementary information

Design, synthesis, and cell-based in vitro assay of deoxyinosine-mixed SATE-dCDN prodrugs that activate all common STING variants

Zhiqiang Xie [a], Yuchen Yang [a], Dejun Ma [a], Zhen Xi *[, a, b, c, d]

[a.] State key Laboratory of Elemento-organic Chemistry, College of Chemistry, Nankai University, Tianjin 300071, China

[b.] National Pesticide Engineering Research Centre (Tianjin 300071)

[c.] Haihe Laboratory of Sustainable Chemical Transformations, Tianjin 300192 (China)

[d.] Collaborative Innovation Centre of Chemical Science and Engineering (Tianjin), Tianjin 300071, China

∗ Corresponding author.
E-mail address: zhenxi@nankai.edu.cn. (Z. Xi)

Table of Contents

1. Serum stability

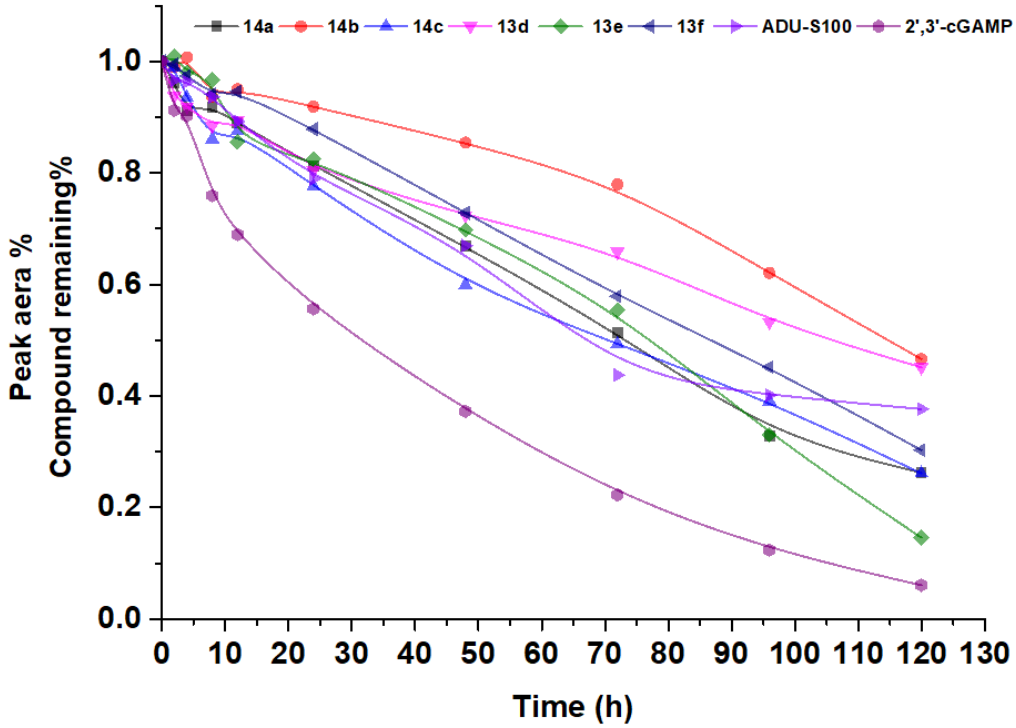


Fig. S1 Deoxyinosine-mixed SATE-dCDN prodrugs retained the high serum stability. The serum stability analysis of prodrugs was determined in 20% FBS. Each compound (100 μM) was incubated in 20 % FBS at 37℃. At various time (0 h, 2 h, 4 h, 8 h, 12 h, 24 h, 48 h, 72 h, 96 h, 120 h), aliquots of the reaction mixture were analyzed with HPLC at 254 nm. The % remaining of test compounds after incubation in serum was calculated.

Table S1. The half-life period of deoxyinosine-mixed dCDN prodrugs and CDNs

Compounds	$T_{1/2}$（h）
13d	105
13e	77
13f	86
14a	73
14b	114
14c	70
15	72
2',3'-cGAMP	31
ADU-S100	67

2.Intracellular uptake and efflux of SATE-dCDN prodrugs

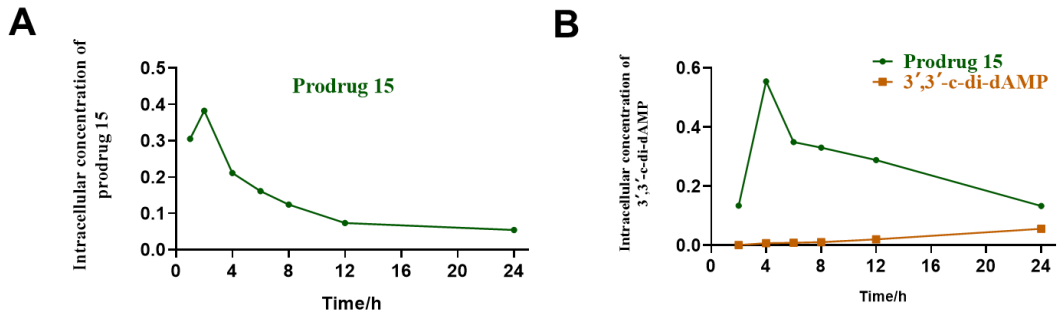


Fig. S2. Uptake and efflux of SATE-dCDN prodrugs. (A) (B) THP-1 cells were treated with 10 μM prodrug **15** or 100 μM parent dCDN 3′,3′-c-di-dAMP for the indicated period of time, and intracellular levels of the metabolites were determined in cellular extracts.

3. Activities of deoxyinosine-mixed dCDN prodrugs and CDNs

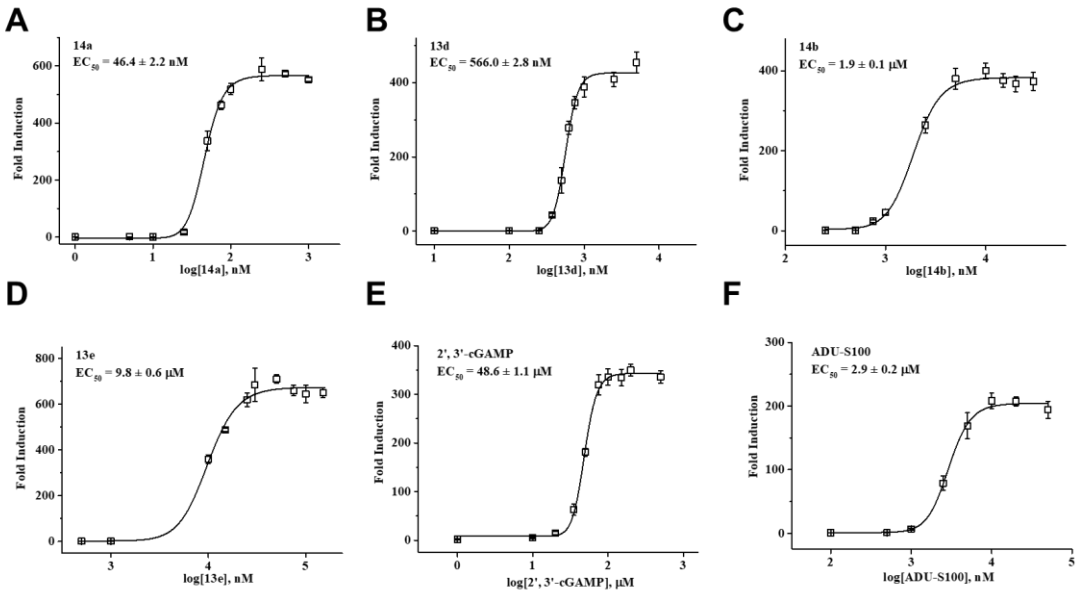


Fig. S3. Activities of deoxyinosine-mixed dCDN prodrugs and CDNs. The EC_{50} values of **14a** (A), **13d** (B), **14b** (c), **13e** (d), 2′,3′-cGAMP (E) and ADU-S100 (F) were also assessed in THP1-Lucia cells.

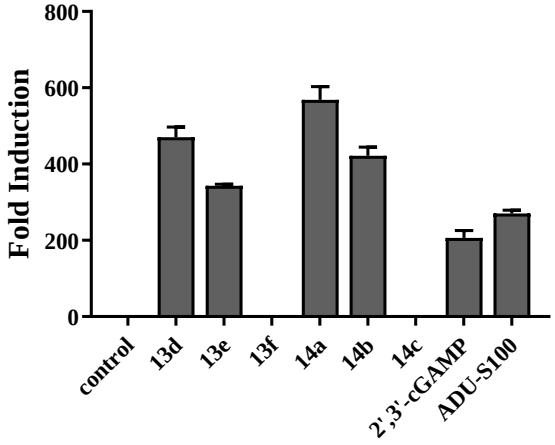


Fig. S4. The comparison of E_{max}. The E_{max} values of **13d-13e**, **14a-14c**, 2′,3′-cGAMP and ADU-S100 were also assessed in THP1-Lucia cells.

4. Cell viability

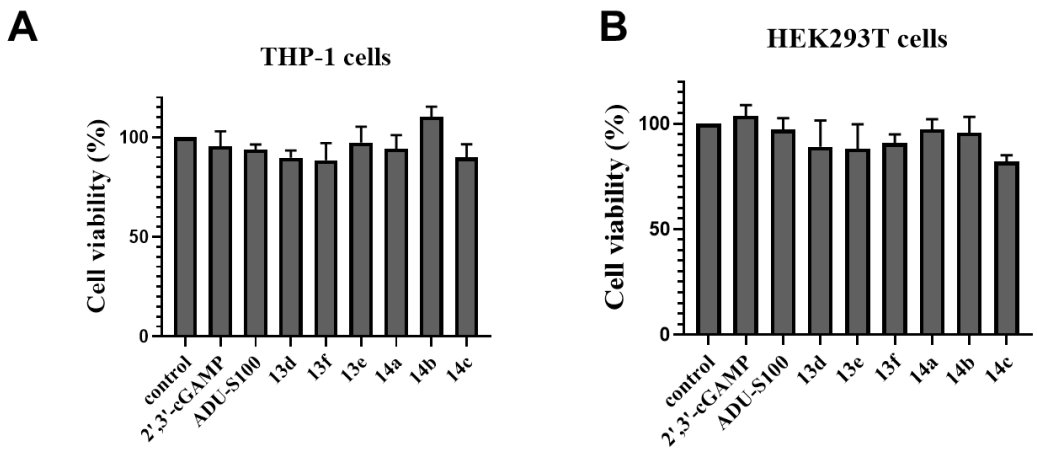


Fig. S5. Cell viability of THP-1 and HEK293T cells stimulated by inosine-mixed dCDN prodrugs and CDNs. (A) 5×10^3 HEK293T cells or (B) THP-1 cells were seeded in 96-well plates. Cells were stimulated with inosine-mixed dCDN prodrugs (10 μM), 2′,3′-cGAMP (100 μM) and ADU-S100 (10 μM) for 24 h. Then Cell Counting Kit-8 (CCK8) (US EVERBRIGHT, c6005) was used to quantitatively assess cell viability through the OD value at 450 nm. Data were presented as mean ± SD, n = 3.

5. Activity against five hSTING variants

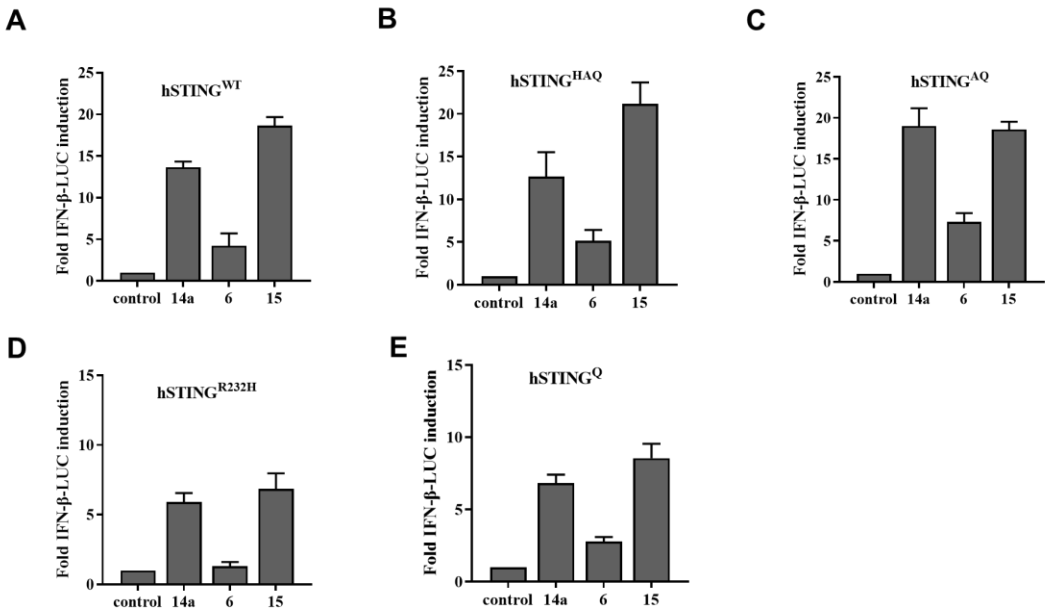


Fig. S6. The luciferase-based bioactivity evaluation of **14a**, 3′,3′-c-di-dIdAMP (**6**), SATE-3′,3′-c-di-dAMP (**15**) against five hSTING variants. HEK293T cells were cotransfected with IFN-β-luciferase reporter plasmids and the test plasmids expressing (A) hSTING-WT, (B) hSTING-HAQ, (C) hSTING-AQ, (D) hSTING-R232H, and (E) hSTING-R293Q. After 24 h, cells were further stimulated for 24 h in the presence of tested compound (50 μM). The cells were lysed for luciferase assay. Data were presented as mean ± SD, n=3.

6. RT-qPCR primers of target genes and the internal control gene

Table S2. RT-qPCR primers of target genes and the internal control gene

Primer name	Sequence (5'-3')
IFN-β-F	AACAAGTGTCTCCTCCAAAT
IFN-β-R	TCTCCTCAGGGATGTCAAAG
CXCL10-F	CATTCTGATTTGCTGCCTTAT
CXCL10-R	TTGATGGCCTTCGATTCTGG
IL-6-F	AGACAGCCACTCACCTCTTCAG
IL-6-R	TTCTGCCAGTGCCTCTTTGCTG
TNF-α-F	TGAAAGCATGATCCGGGACG
TNF-α-R	AGGCAGAAGAGCGTGGTGGC

GADPH -F	GGACCTGACCTGCCGTCTA
GADPH-R	GAGTGGGTGTCGCTGTTGA

7. Relative quantification of phospho-STING, phospho-TBK1, phospho-IRF3

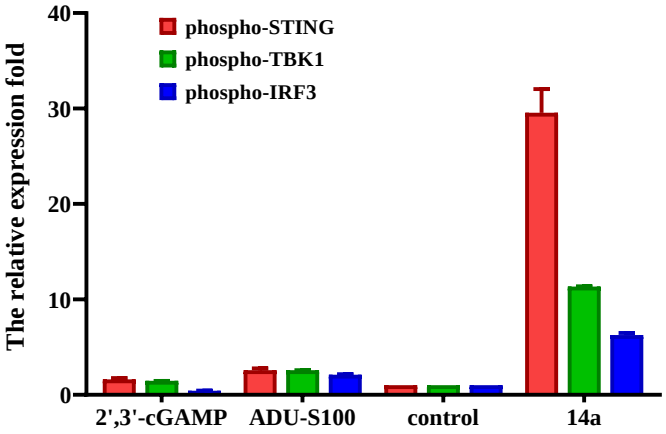
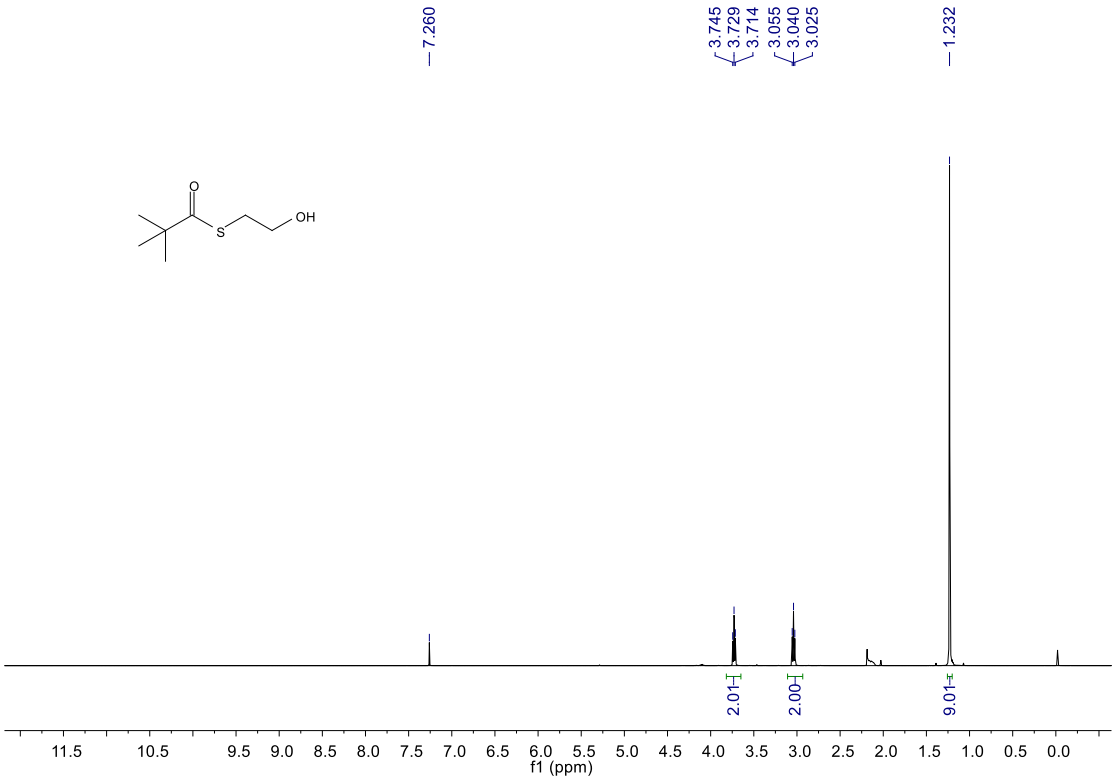
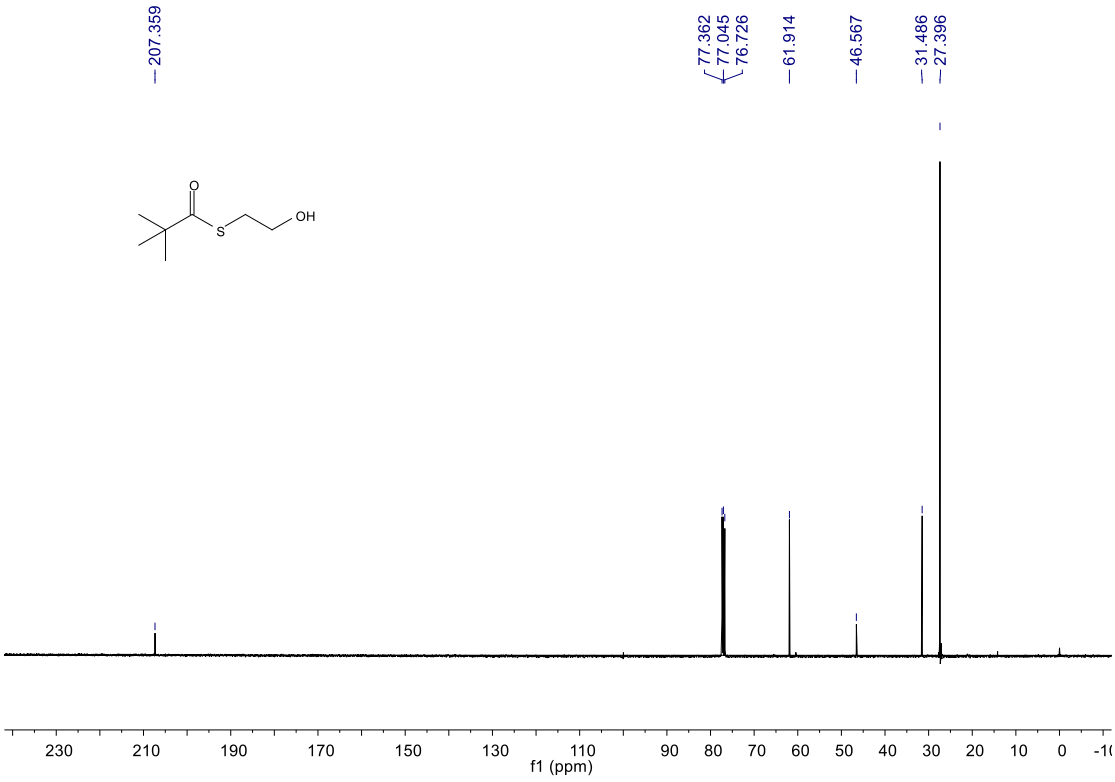


Fig. S7. Relative quantification of phospho-STING, phospho-TBK1, phospho-IRF3. Prodrug **14a** (10 μM), 2′,3′-cGAMP (100 μM) and ADU-S100 (10 μM) were used to treat THP1 cells for 4 h, and the levels of total STING, phospho-STING, total TBK1, phospho-TBK1, total IRF3, phospho-IRF3, and β-actin were assessed by Western blotting.

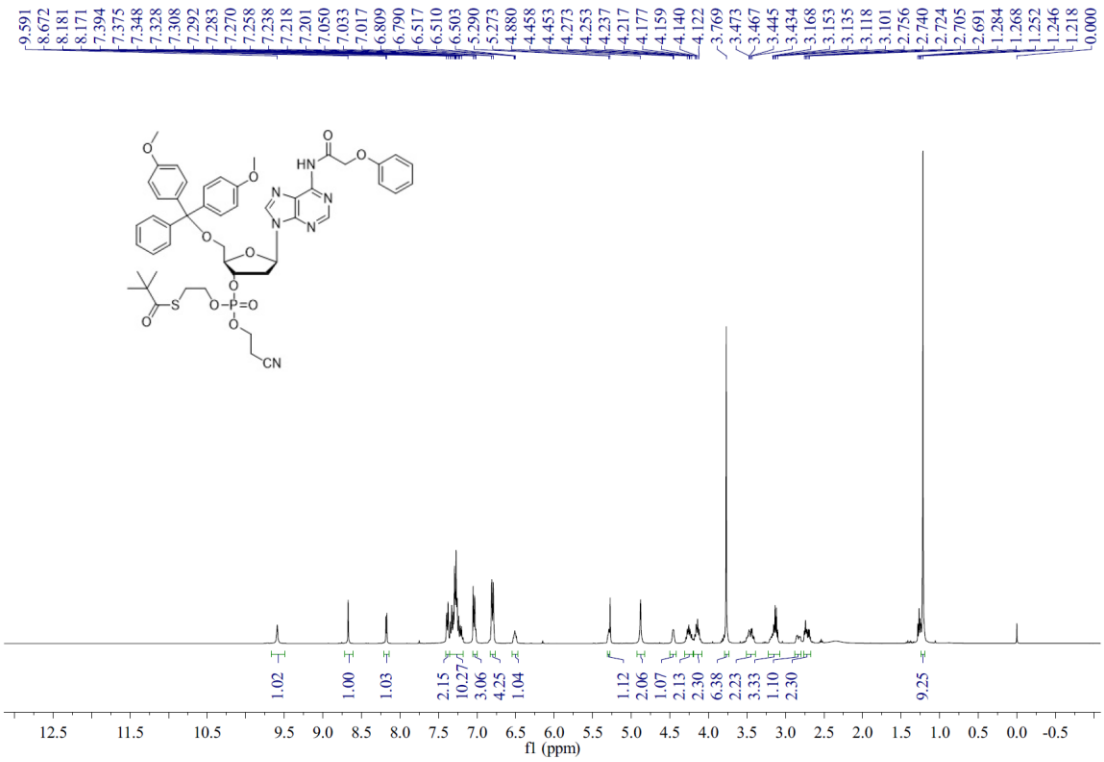
8. Chemical characterization of ^{1}H, ^{13}C, ^{31}P NMR and HPLC spectra

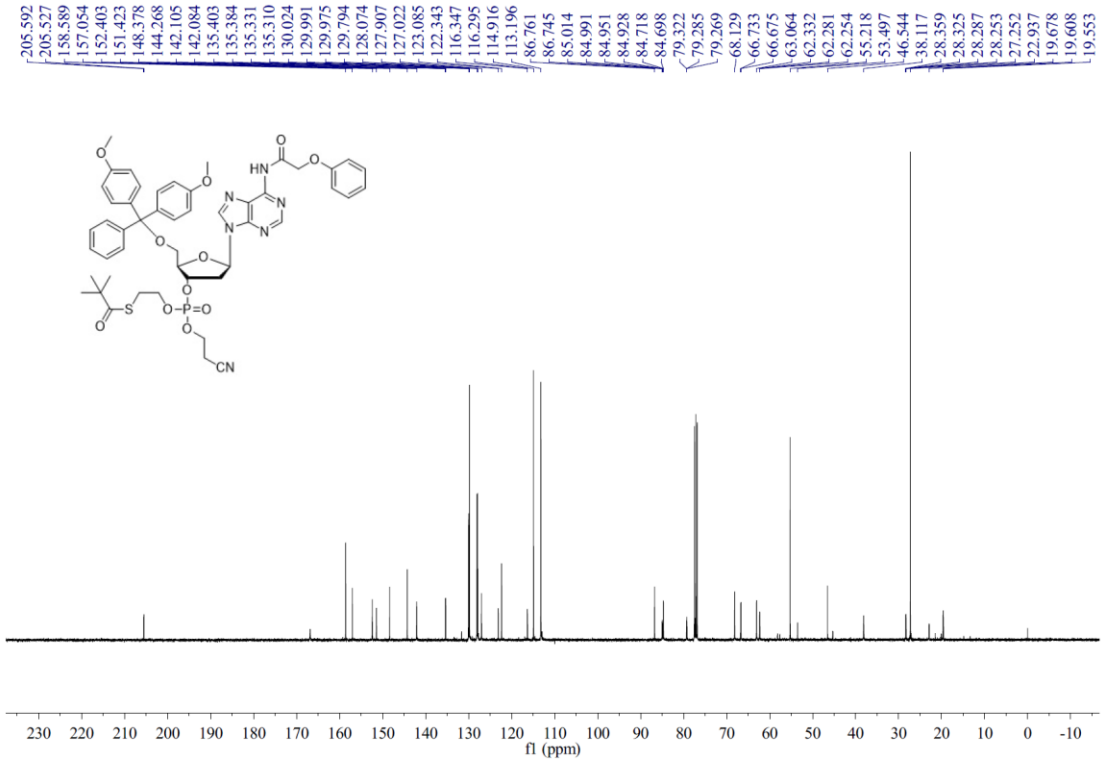
^{1}H, ^{13}C spectra of **8:**

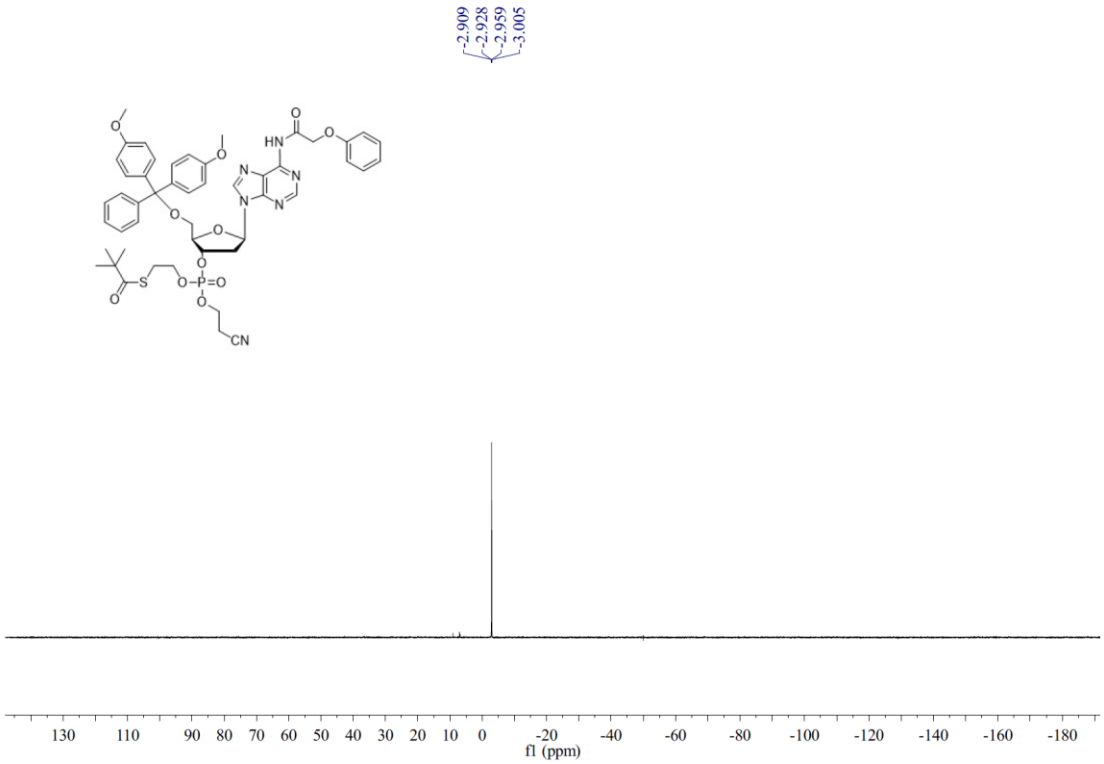




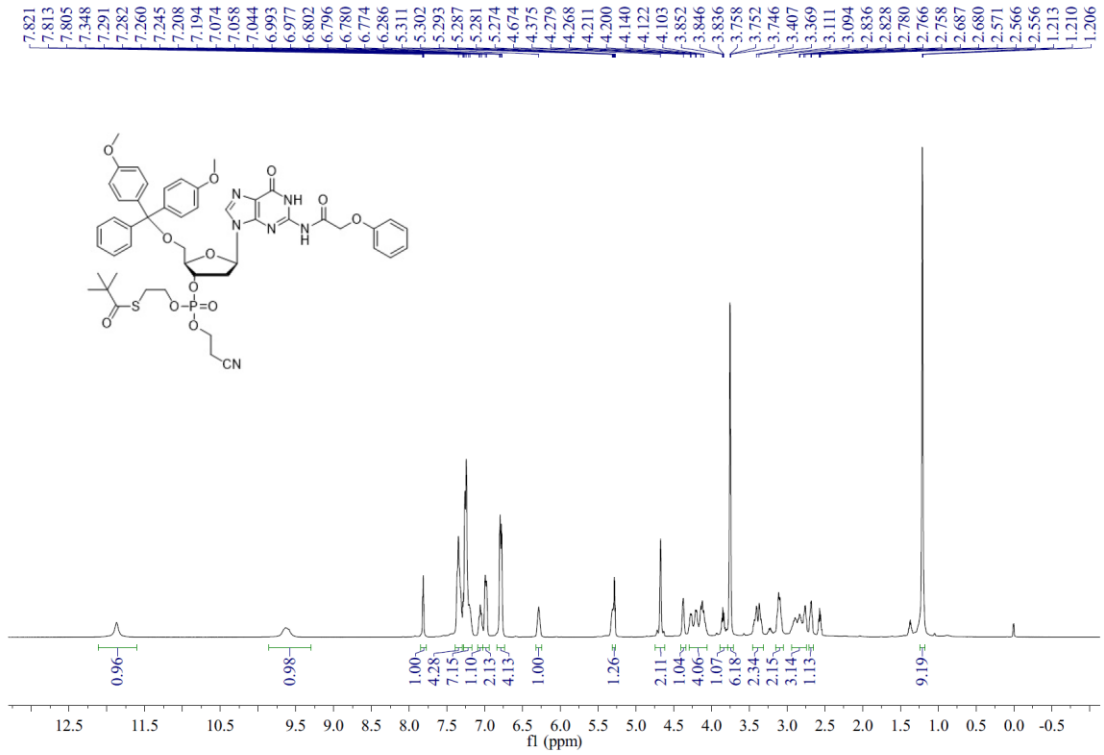
^{1}H, ^{13}C, ^{31}P NMR spectra of **9a:**

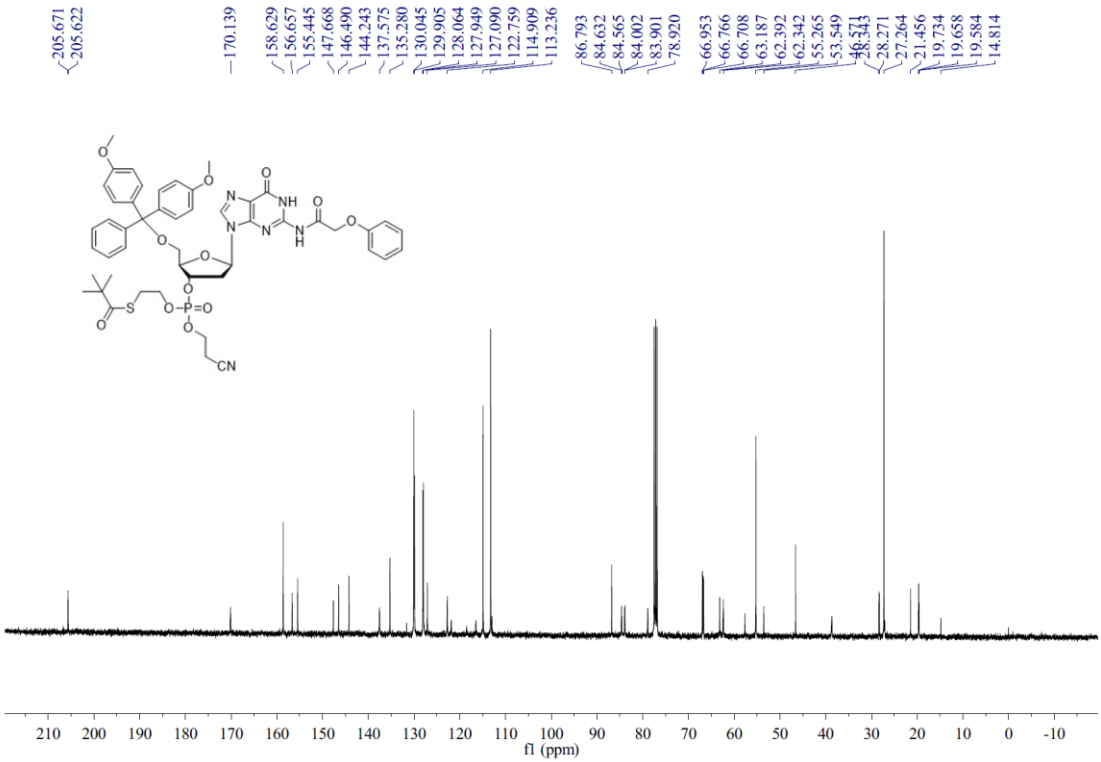


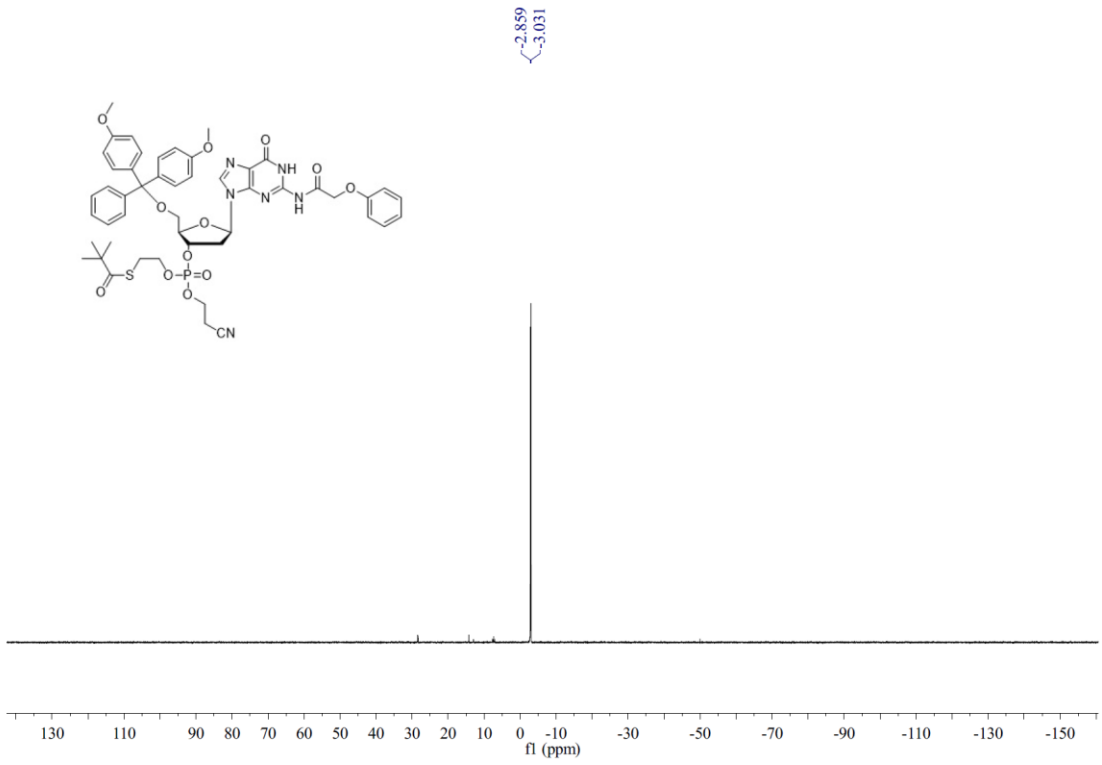




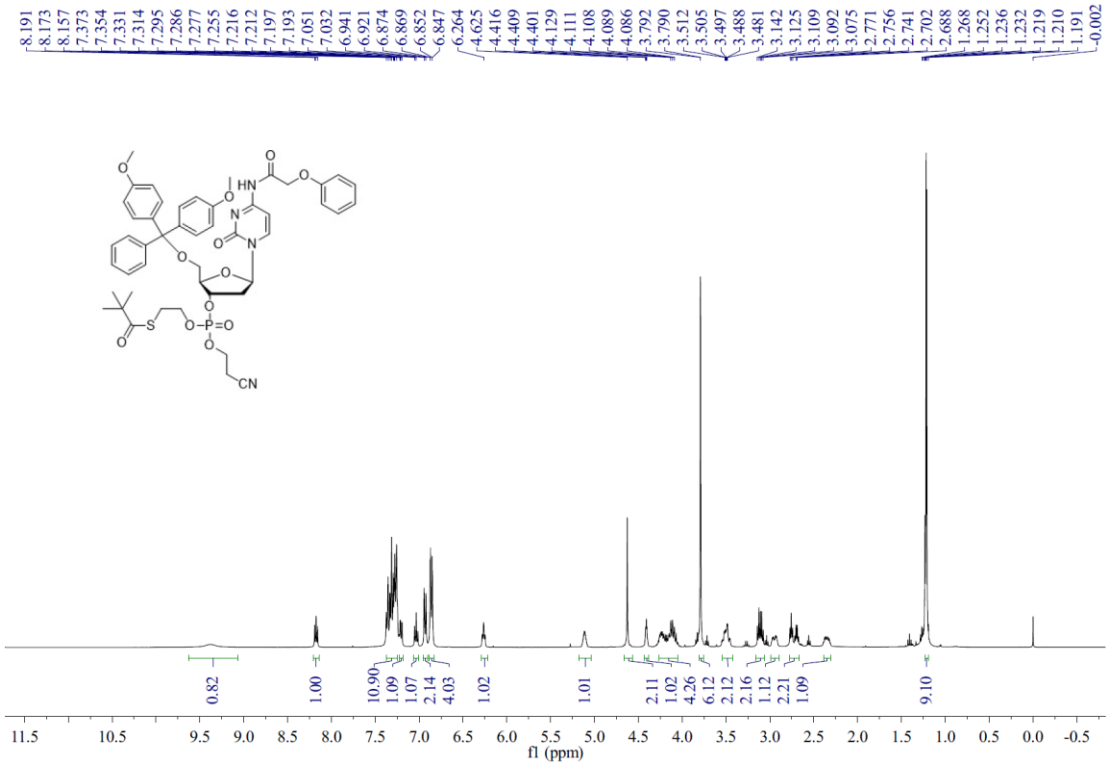
^{1}H, ^{13}C, ^{31}P NMR of **9b:**

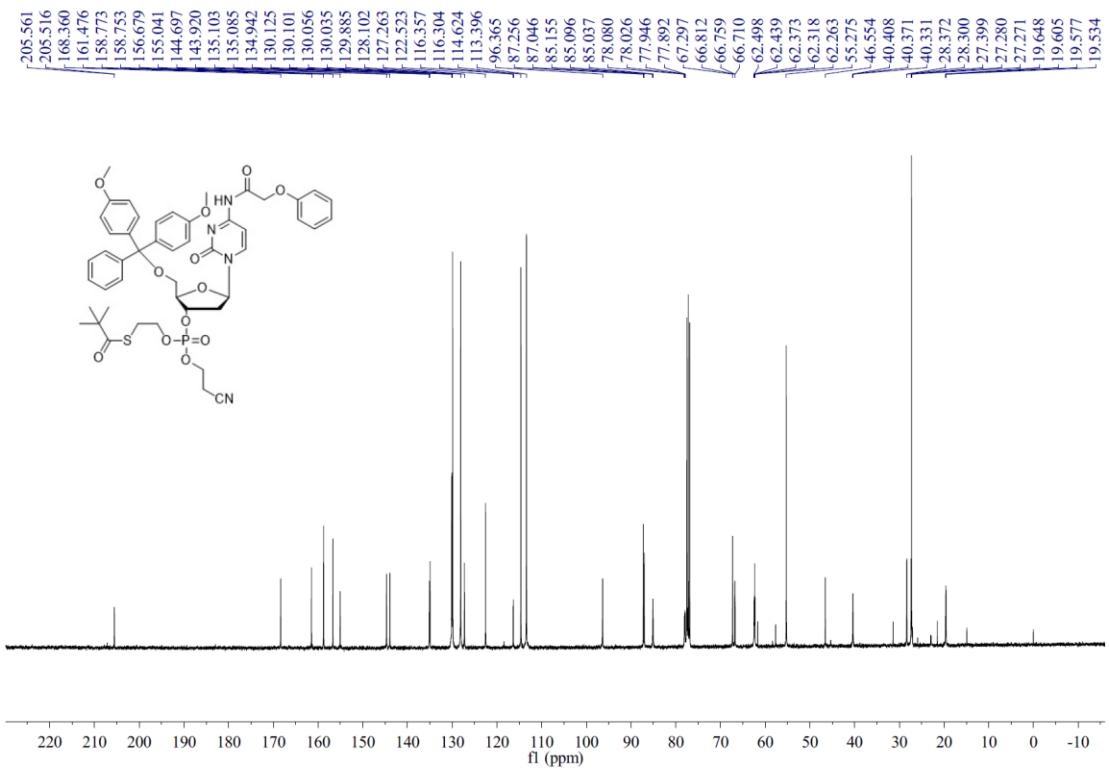


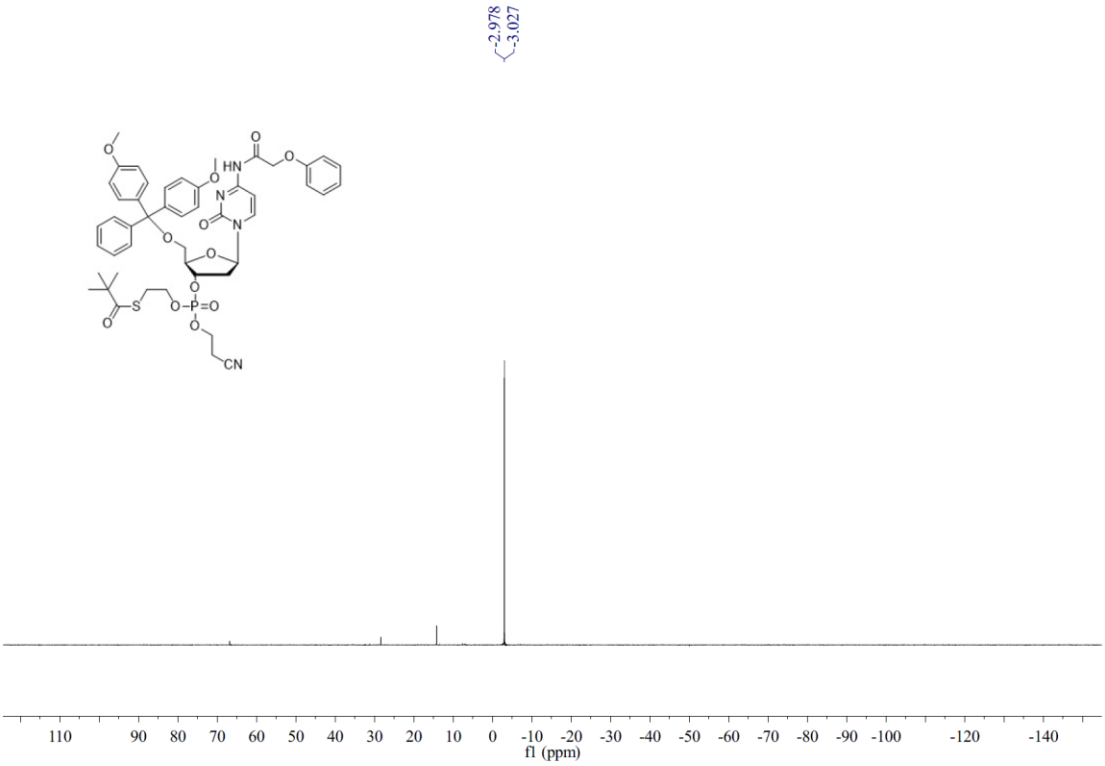

205.671
205.622
170.139
158.629
156.657
155.445
147.668
146.490
144.243
137.575
135.280
130.045
129.905
128.064
127.949
127.090
122.759
114.909
113.236
86.793
84.632
84.565
84.002
83.901
78.920
66.953
66.766
66.708
63.187
62.392
62.342
55.265
53.549
46.341
28.343
28.271
27.264
21.456
19.734
19.658
19.584
14.814
O
O
N
NH
O
N
N
N
H
O
O
O
O
S
O
O
P=O
O
CN
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)


-2.859
-3.031
O
O
N
NH
O
N
N
N
H
O
O
O
O
S
O
O
P=O
O
CN
130 110 90 80 70 60 50 40 30 20 10 0 -10 -30 -50 -70 -90 -110 -130 -150
f1 (ppm)

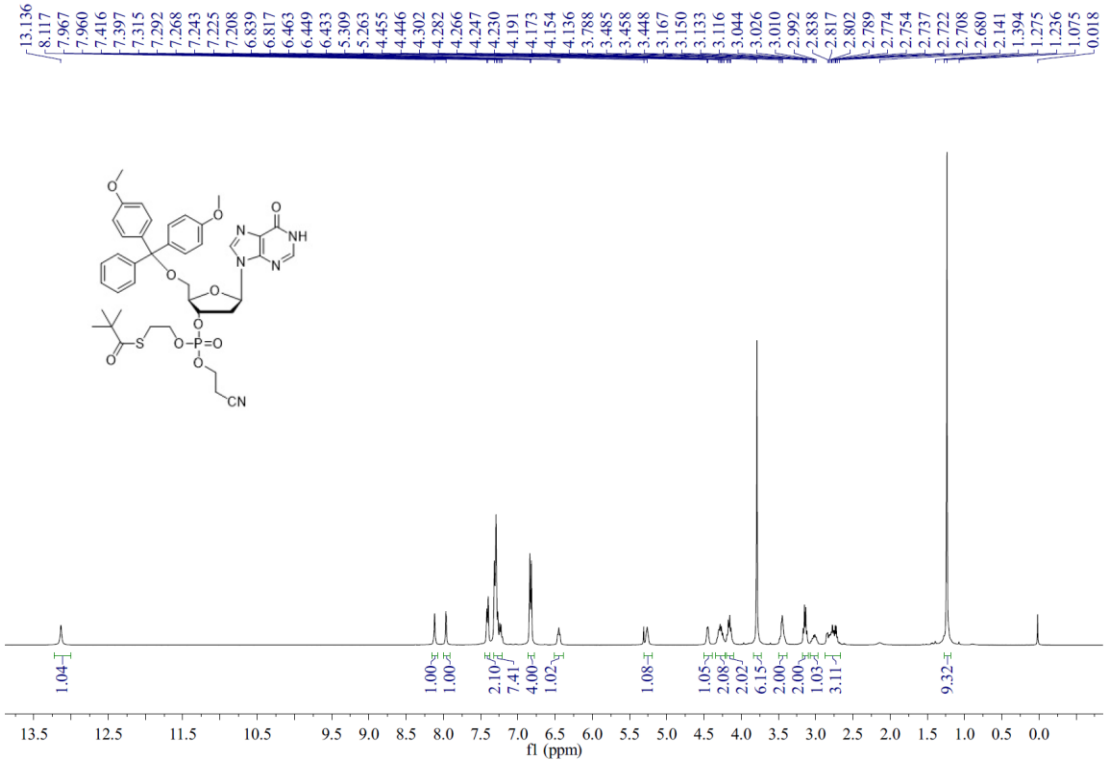
^{1}H, ^{13}C, ^{31}P NMR of **9c:**

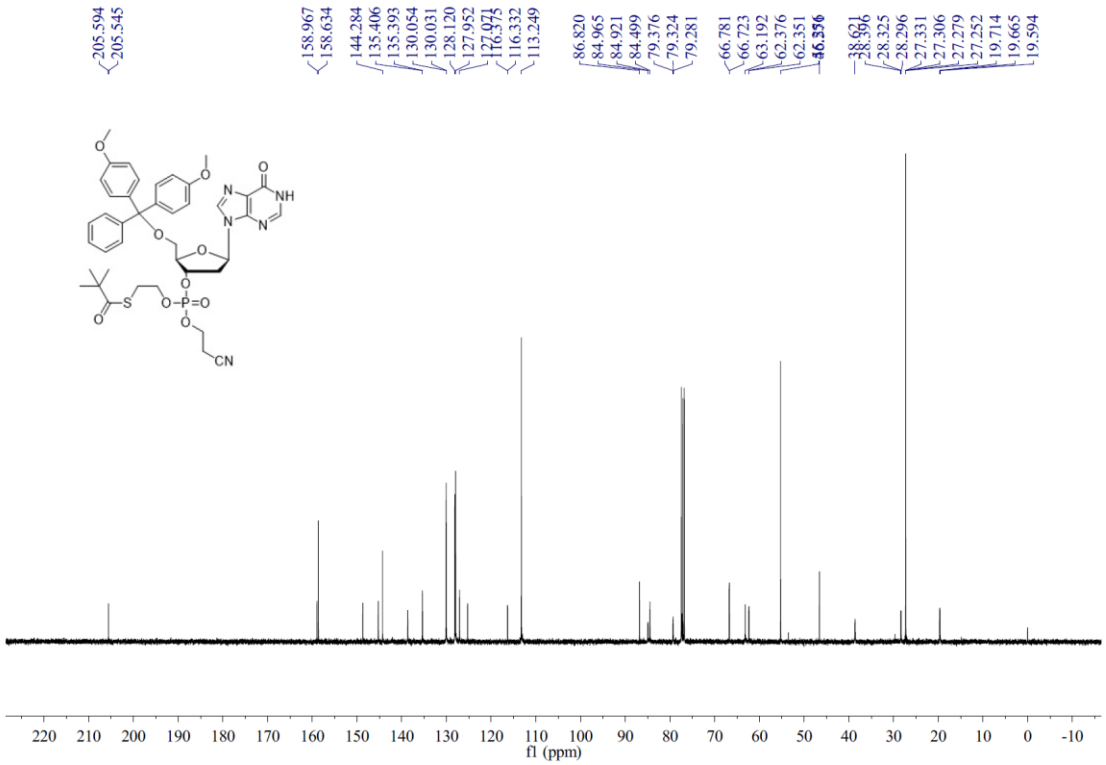


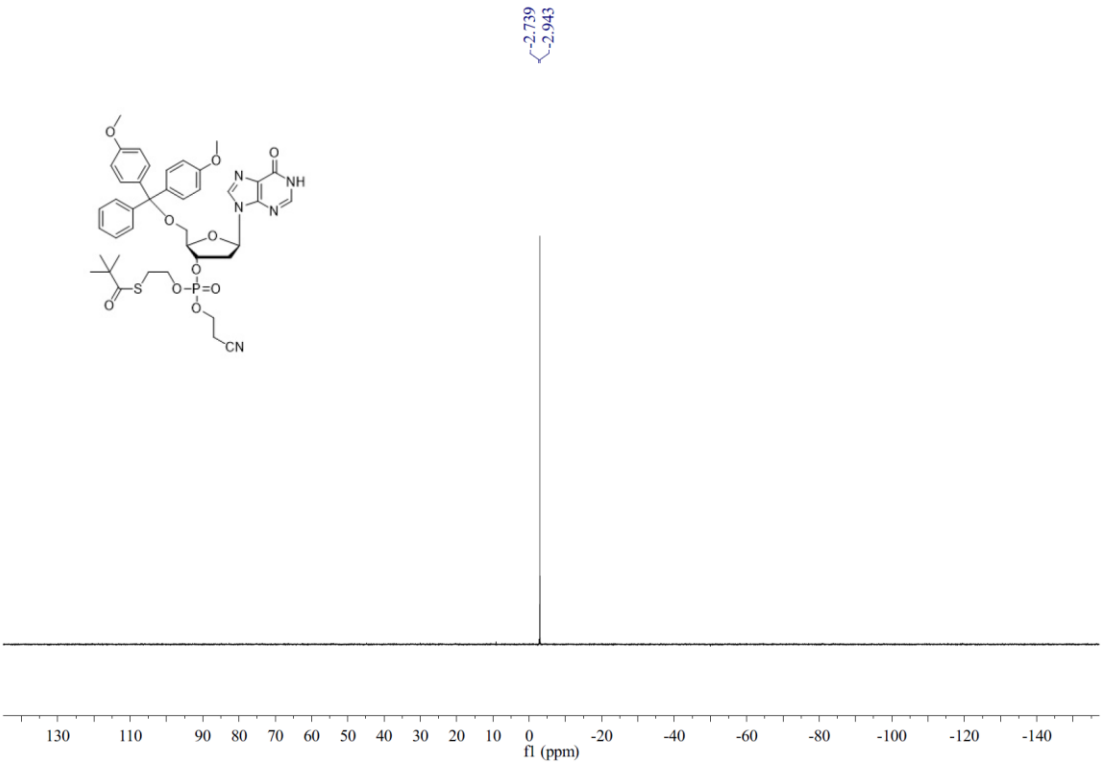




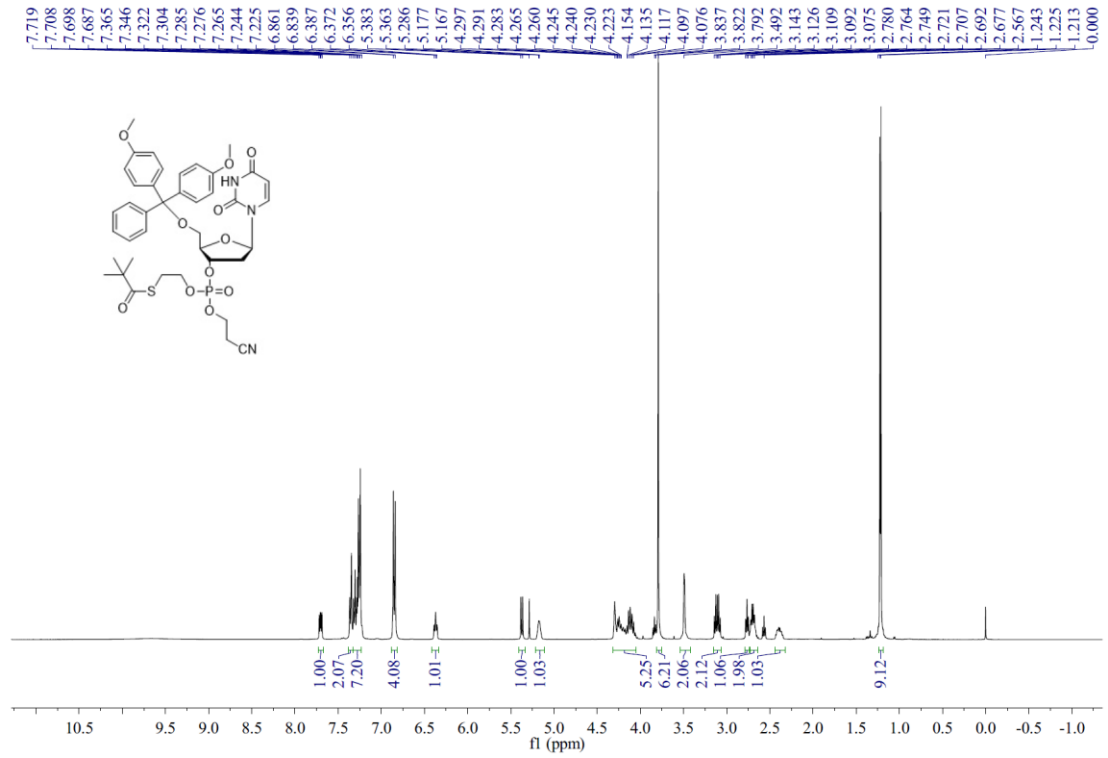
^{1}H, ^{13}C, ^{31}P NMR of **9d:**

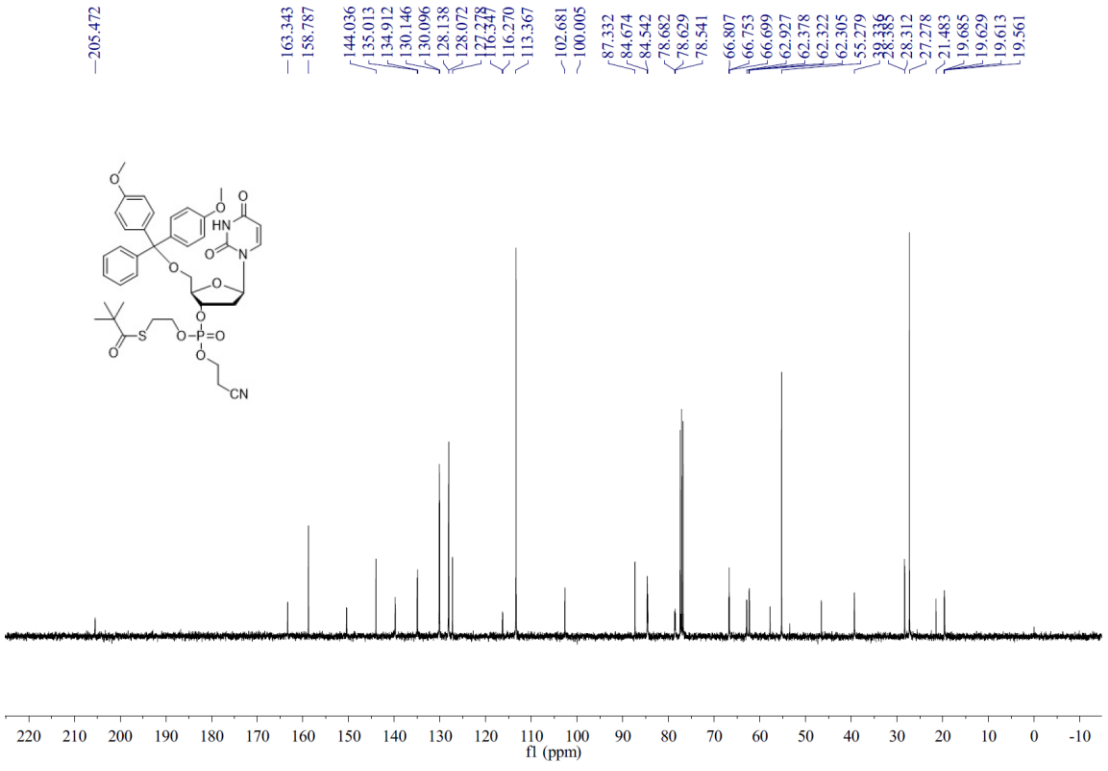


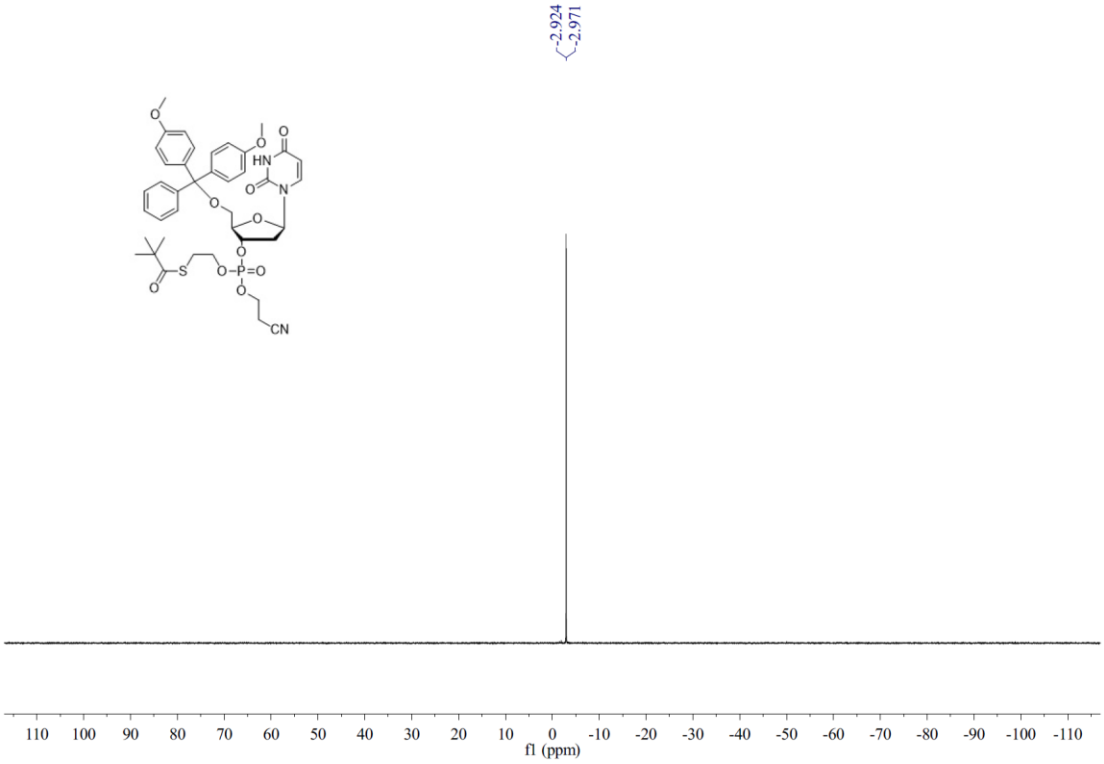

205.594
205.545
158.967
158.634
144.284
135.406
135.393
130.054
130.031
128.120
127.952
116.332
113.249
86.820
84.965
84.921
84.499
79.376
79.324
79.281
66.781
66.723
63.192
62.376
62.351
28.325
28.296
27.331
27.306
27.279
27.252
19.714
19.665
19.594
NH
N
O
S
P
CN
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)


-2.739
-2.943
NH
N
O
S
P
CN
130 110 90 80 70 60 50 40 30 20 10 0 -20 -40 -60 -80 -100 -120 -140
f1 (ppm)

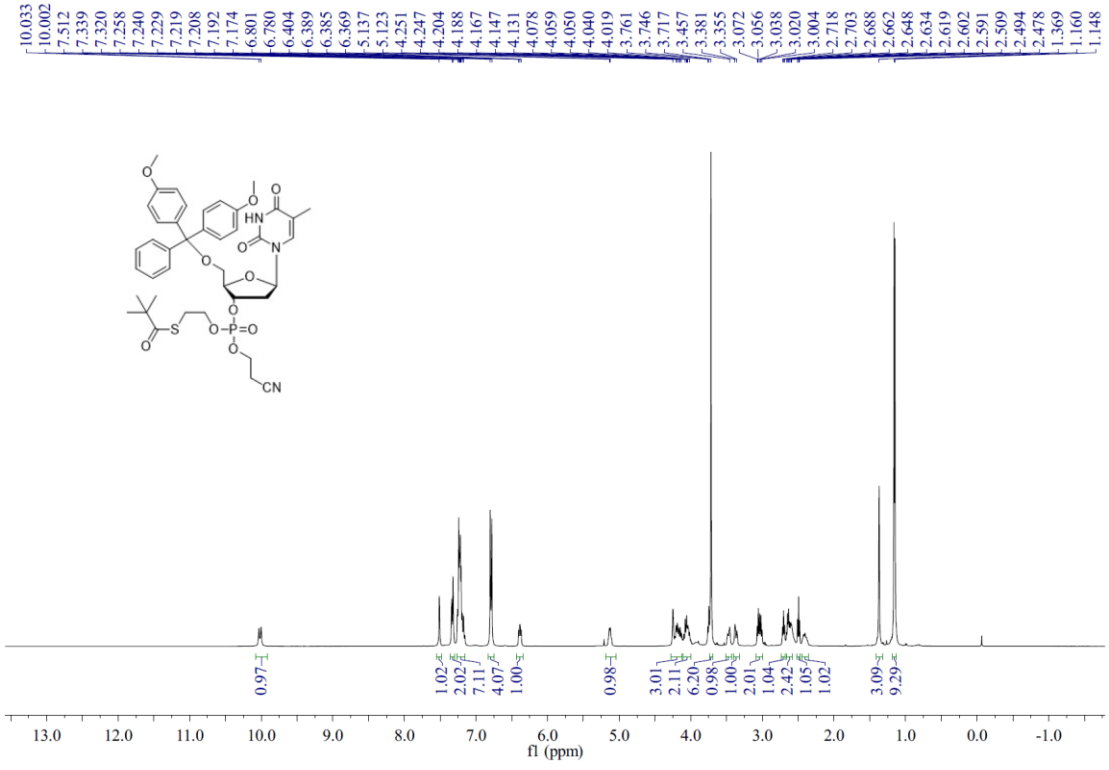
^{1}H, ^{13}C, ^{31}P NMR of **9e:**

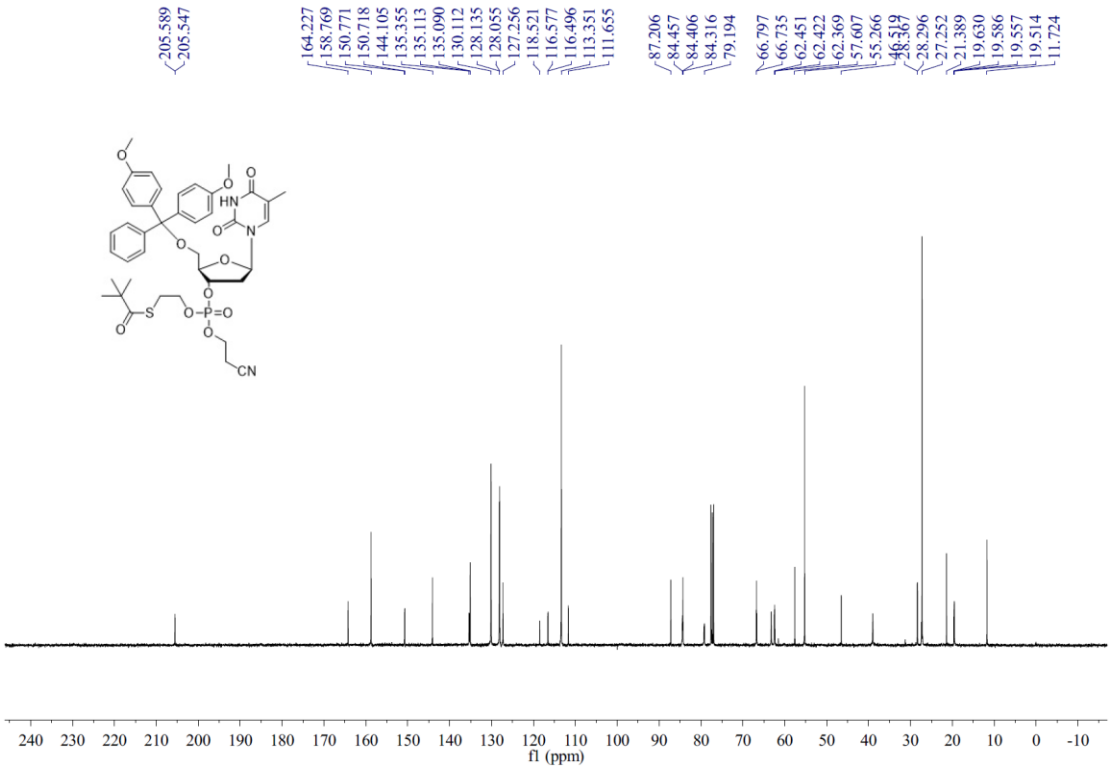


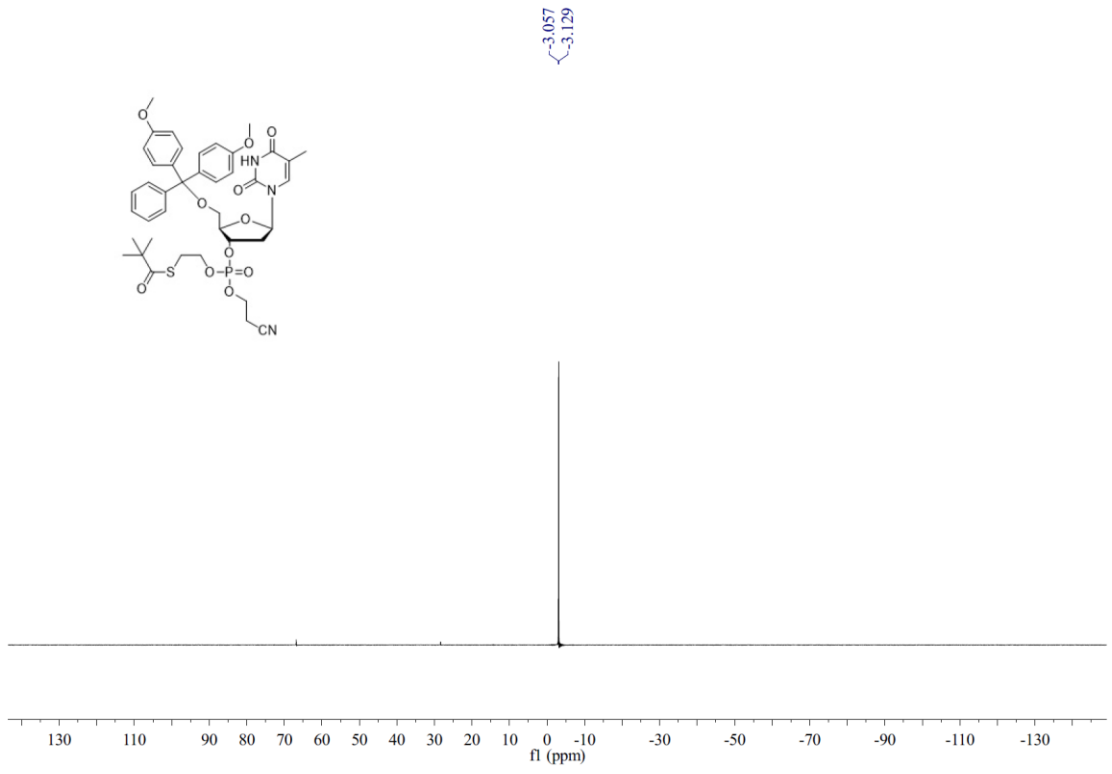




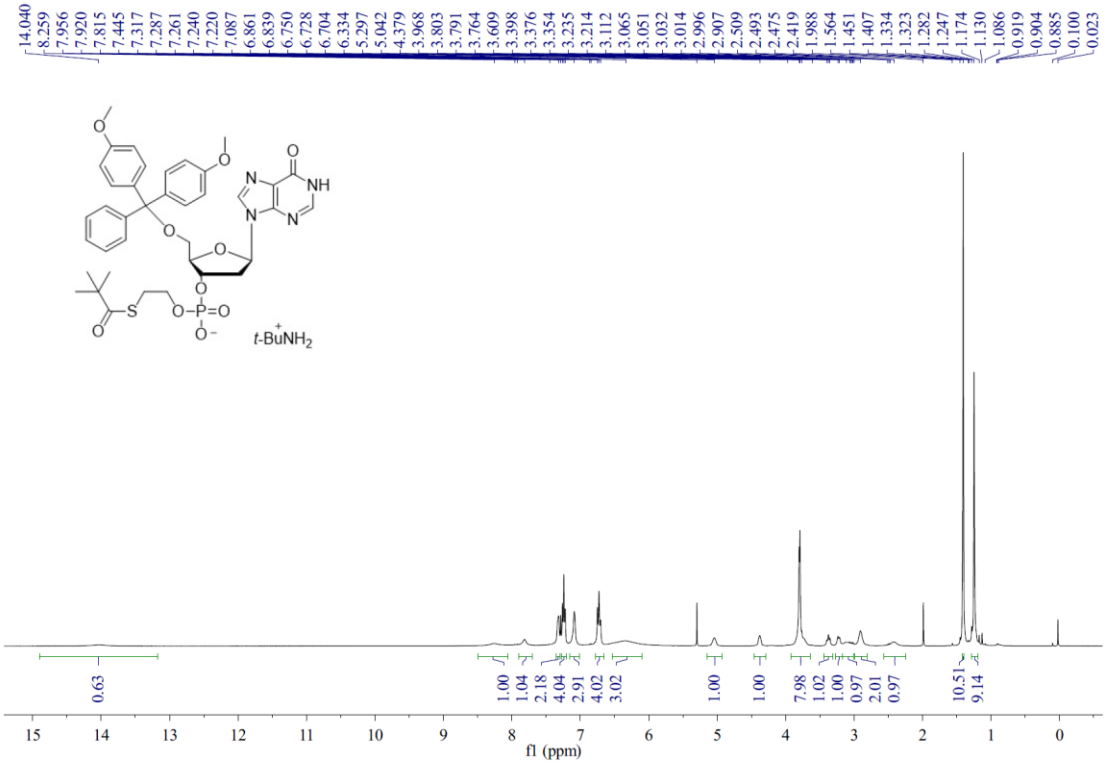
^{1}H, ^{13}C, ^{31}P NMR of **9f:**

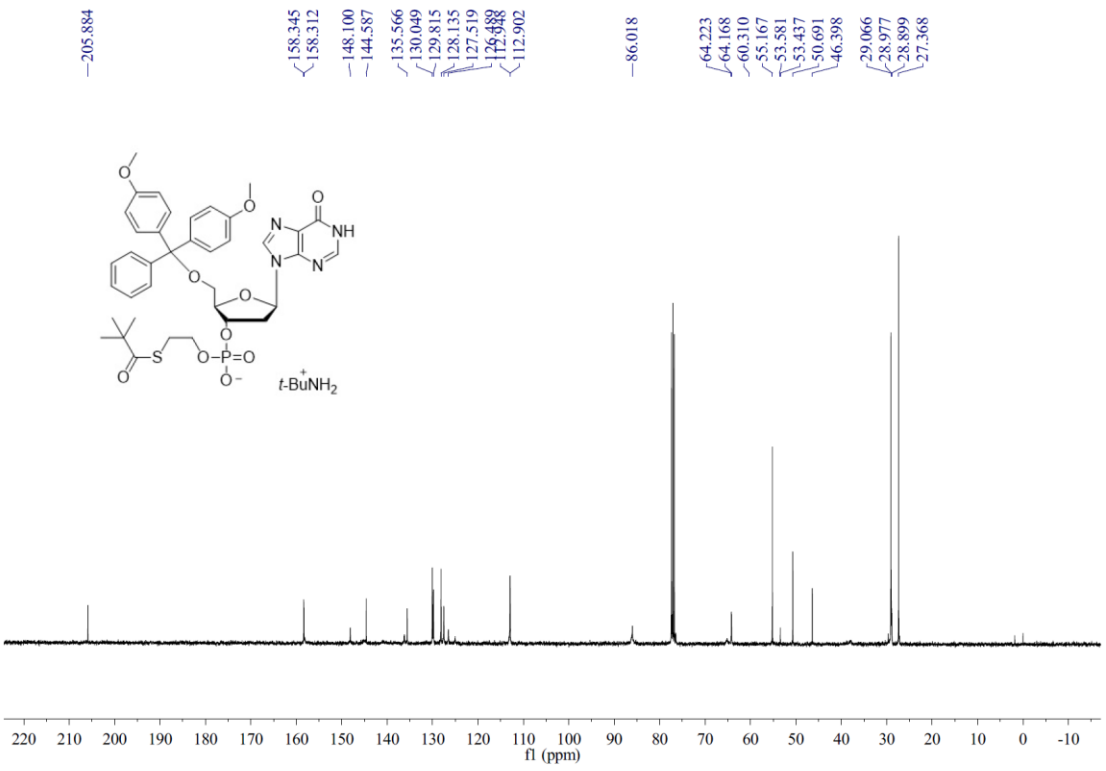


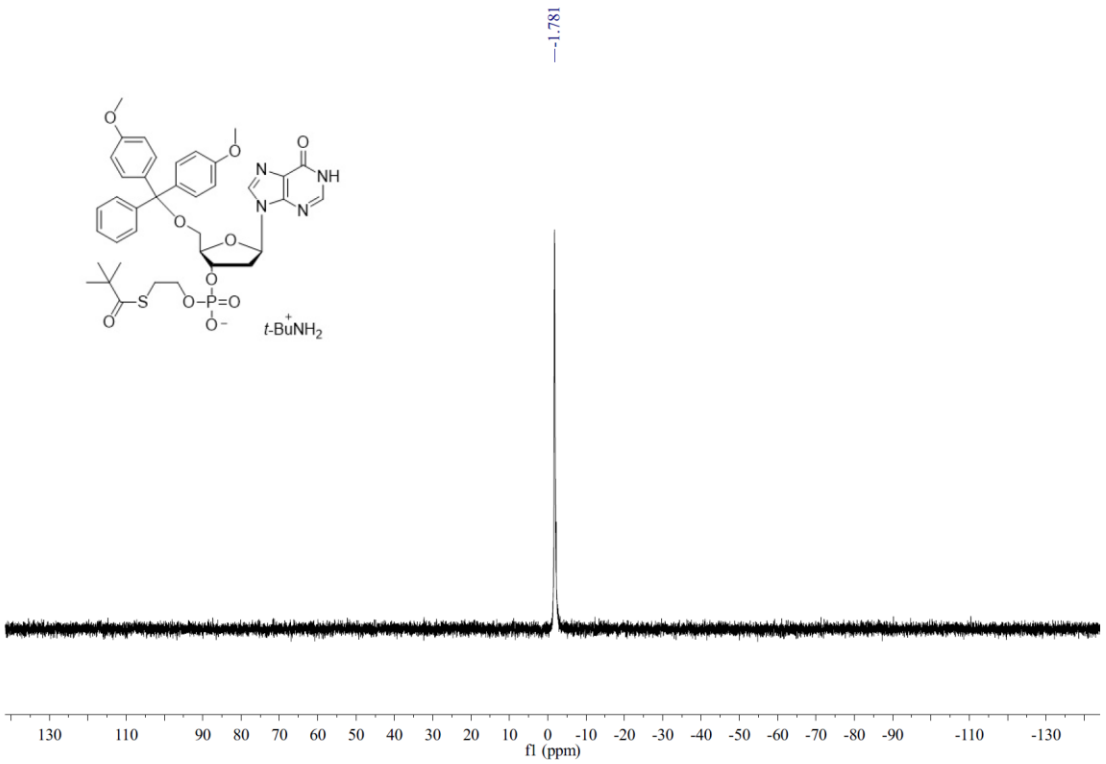

205.589
205.547
164.227
158.769
150.771
150.718
144.105
135.355
135.113
135.090
130.112
128.135
128.055
127.256
118.521
116.577
116.496
113.351
111.655
87.206
84.457
84.406
84.316
79.194
66.797
66.735
62.451
62.422
62.369
57.607
55.266
46.519
46.367
28.296
27.252
21.389
19.630
19.586
19.557
19.514
11.724
HN
O
N
O
O
O
O
O
O
O
S
O-P=O
O
CN
240 230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)


-3.057
-3.129
HN
O
N
O
O
O
O
O
O
O
S
O-P=O
O
CN
130 110 90 80 70 60 50 40 30 20 10 0 -10 -30 -50 -70 -90 -110 -130
f1 (ppm)

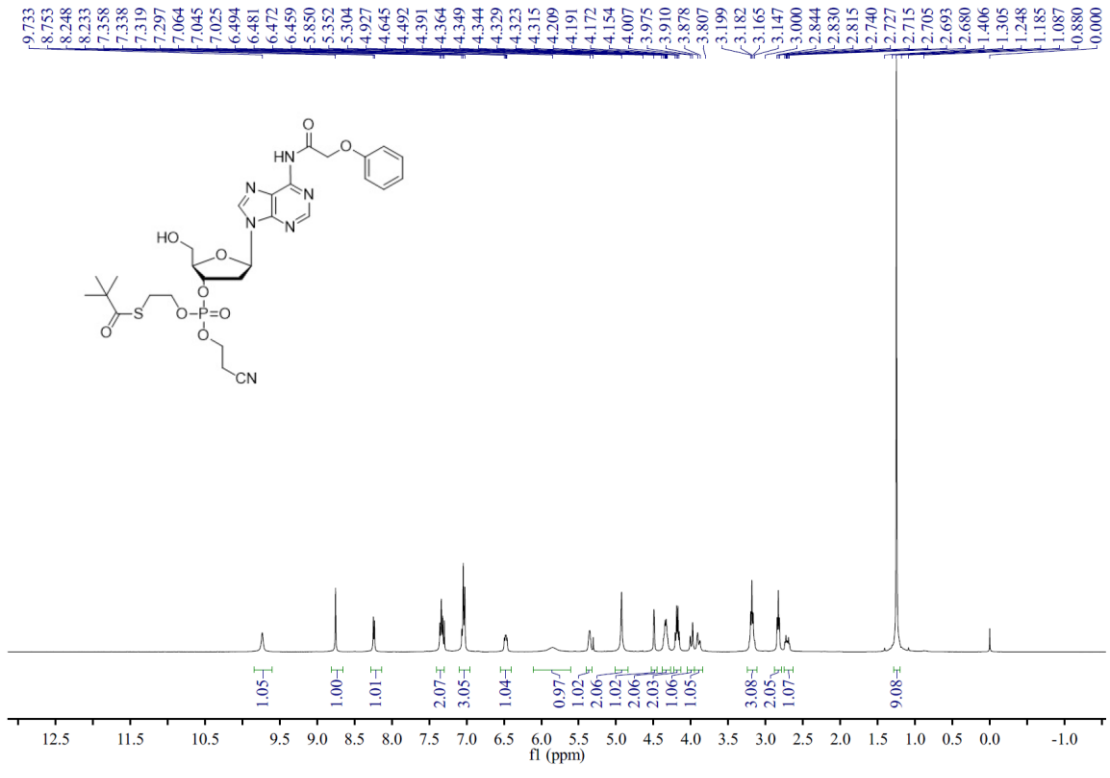
^{1}H, ^{13}C, ^{31}P NMR of **10:**

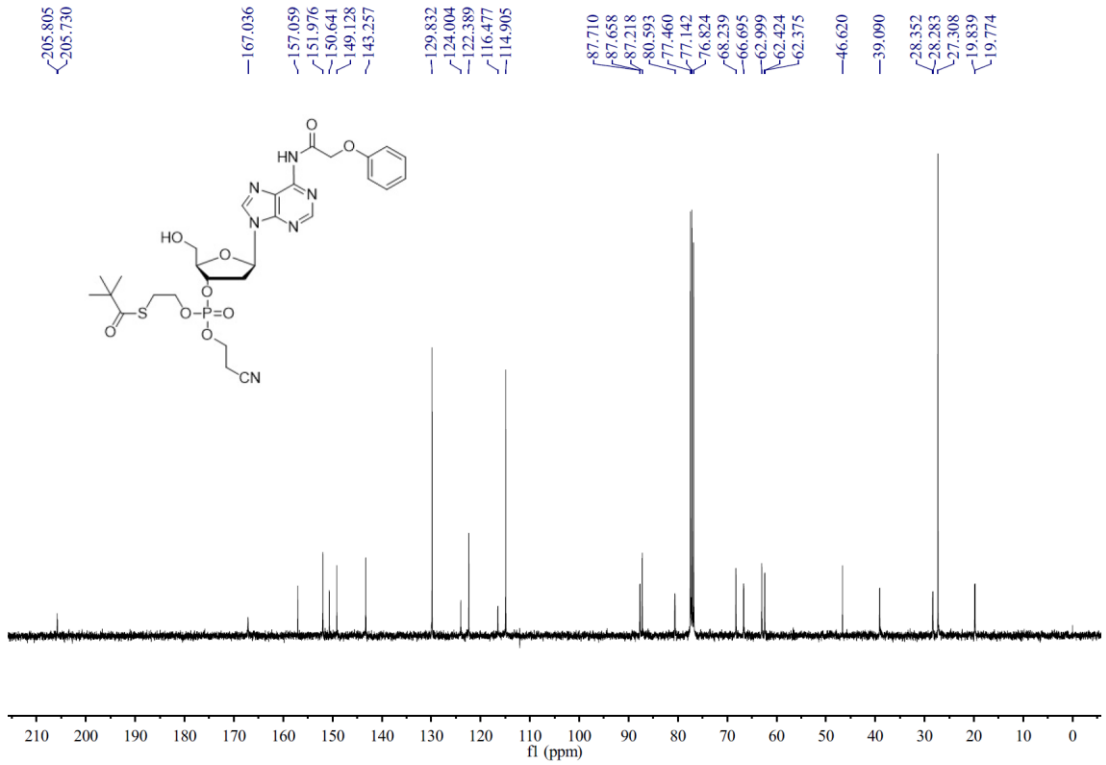


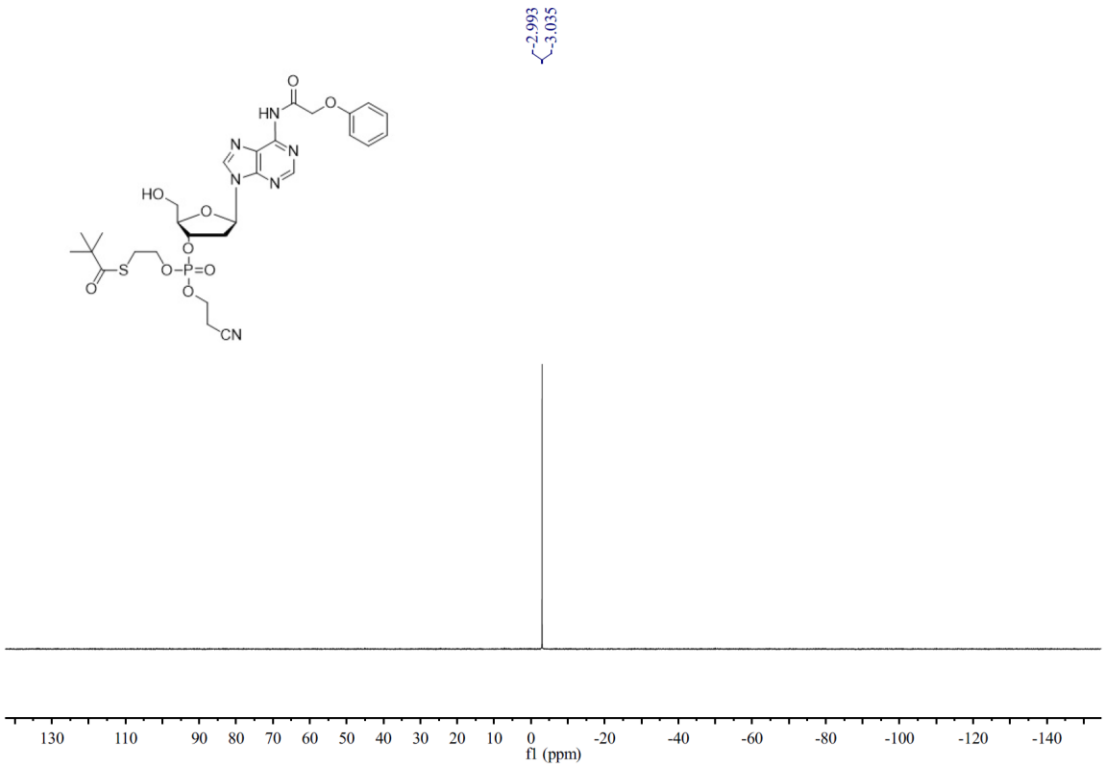




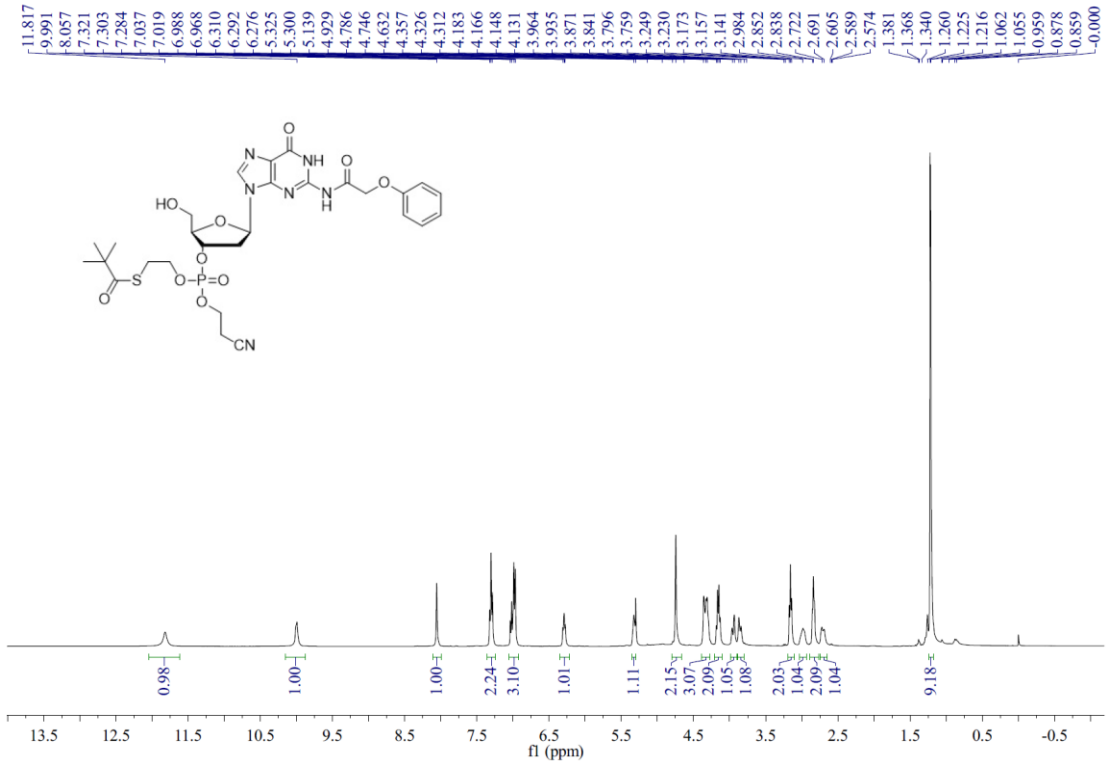
^{1}H, ^{13}C, ^{31}P NMR of **11a:**

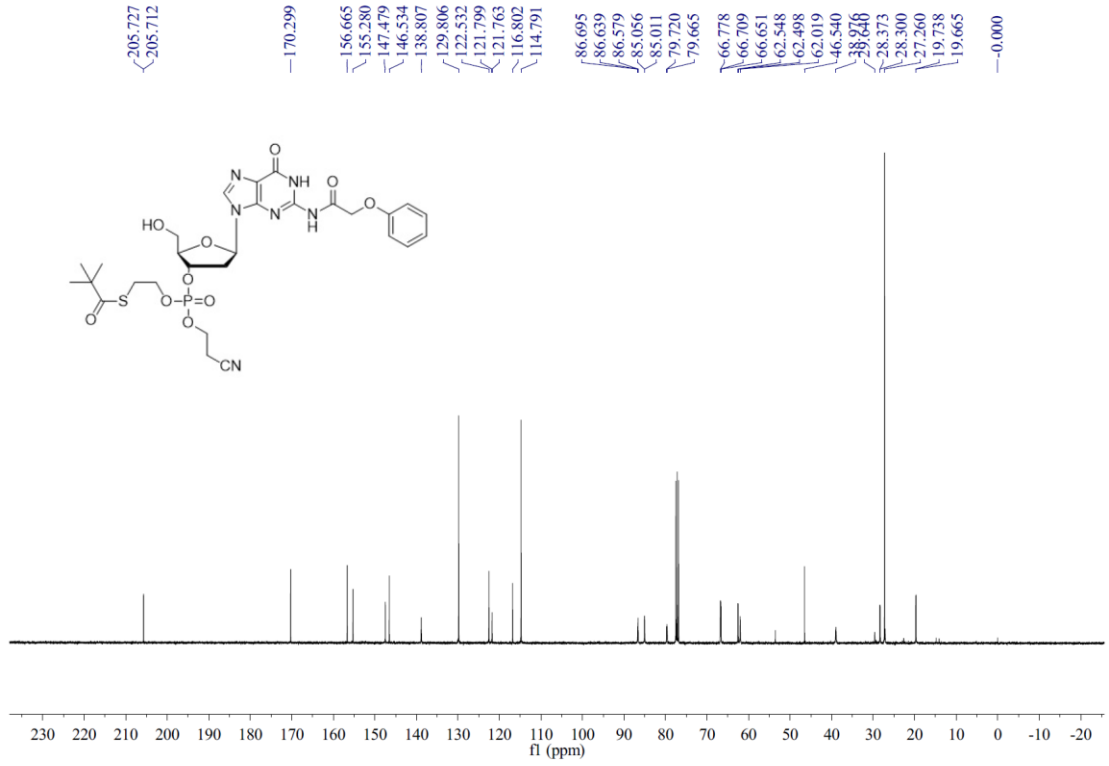


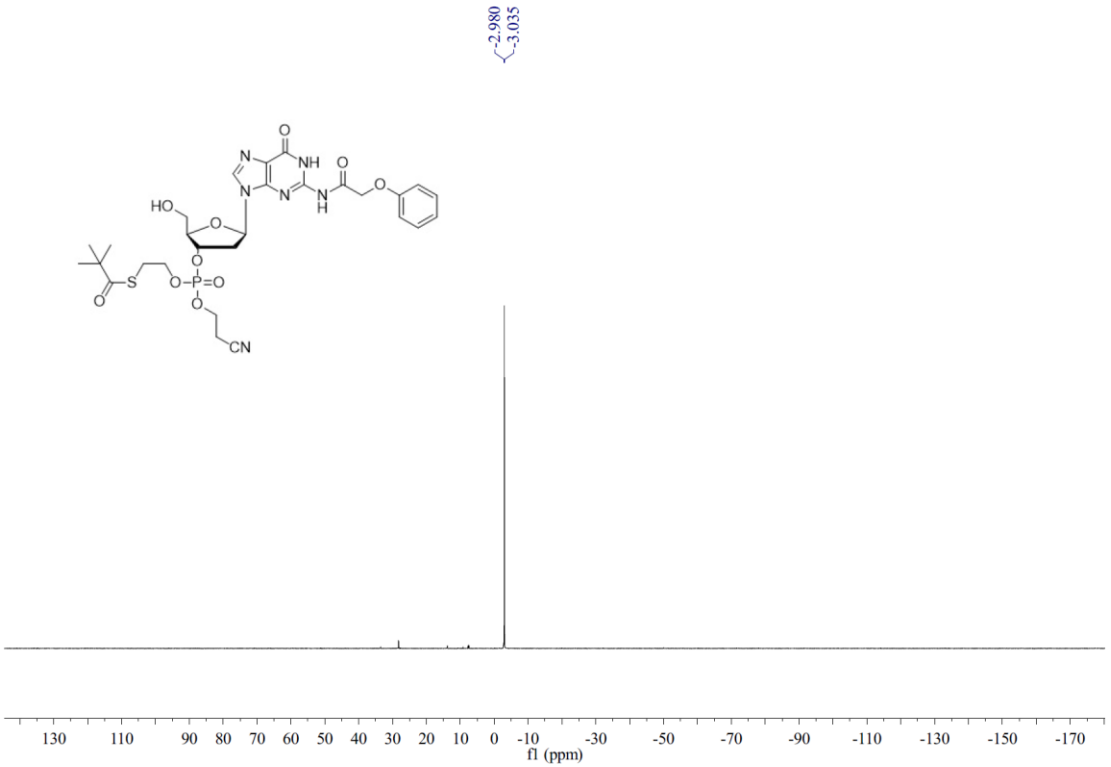

205.805
205.730
167.036
157.059
151.976
150.641
149.128
143.257
129.832
124.004
122.389
116.477
114.905
87.710
87.658
87.218
80.593
77.460
77.142
76.824
68.239
66.695
62.999
62.424
62.375
46.620
39.090
28.352
28.283
27.308
19.839
19.774
HO
HN
O
N
S
O-P=O
CN
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)


2.993
3.035
HO
HN
O
N
S
O-P=O
CN
130 110 90 80 70 60 50 40 30 20 10 0 -20 -40 -60 -80 -100 -120 -140
f1 (ppm)

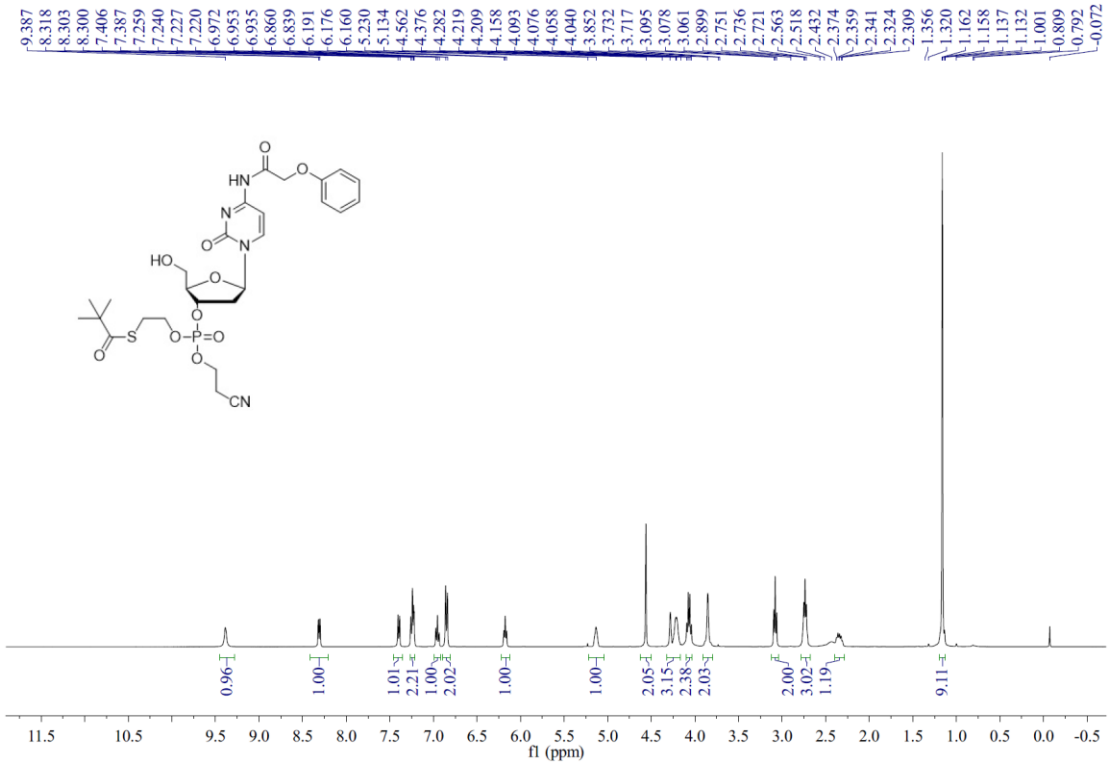
^{1}H, ^{13}C, ^{31}P NMR of **11b:**

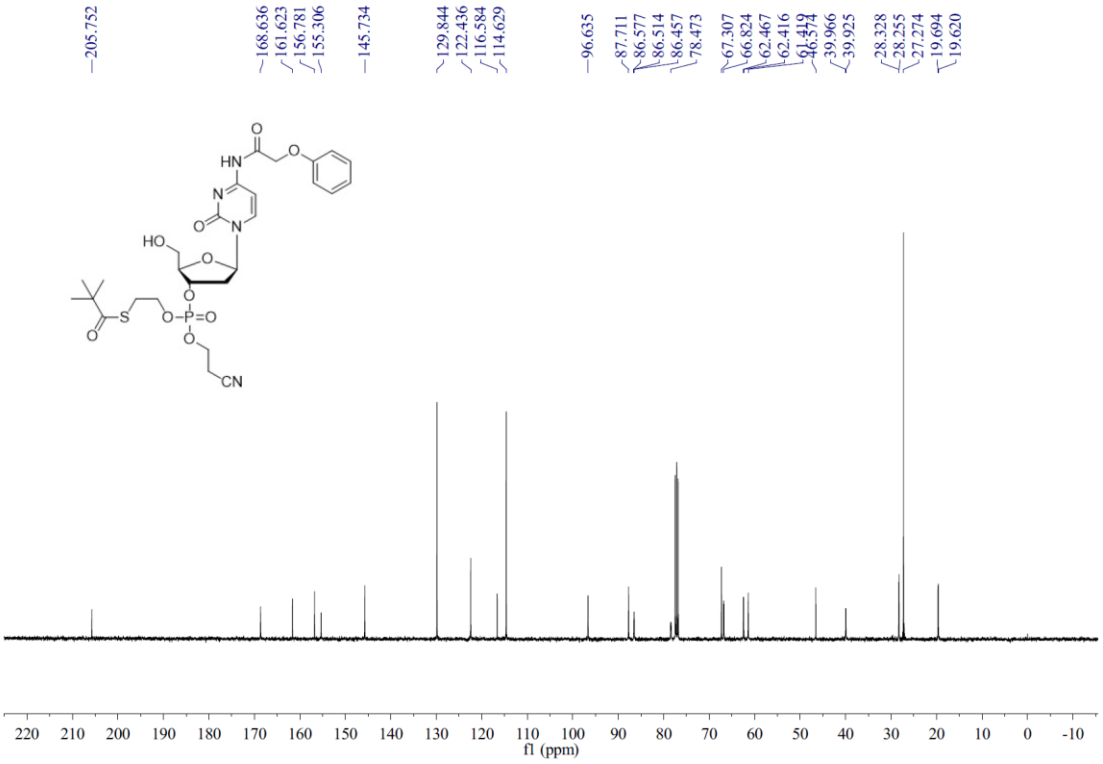


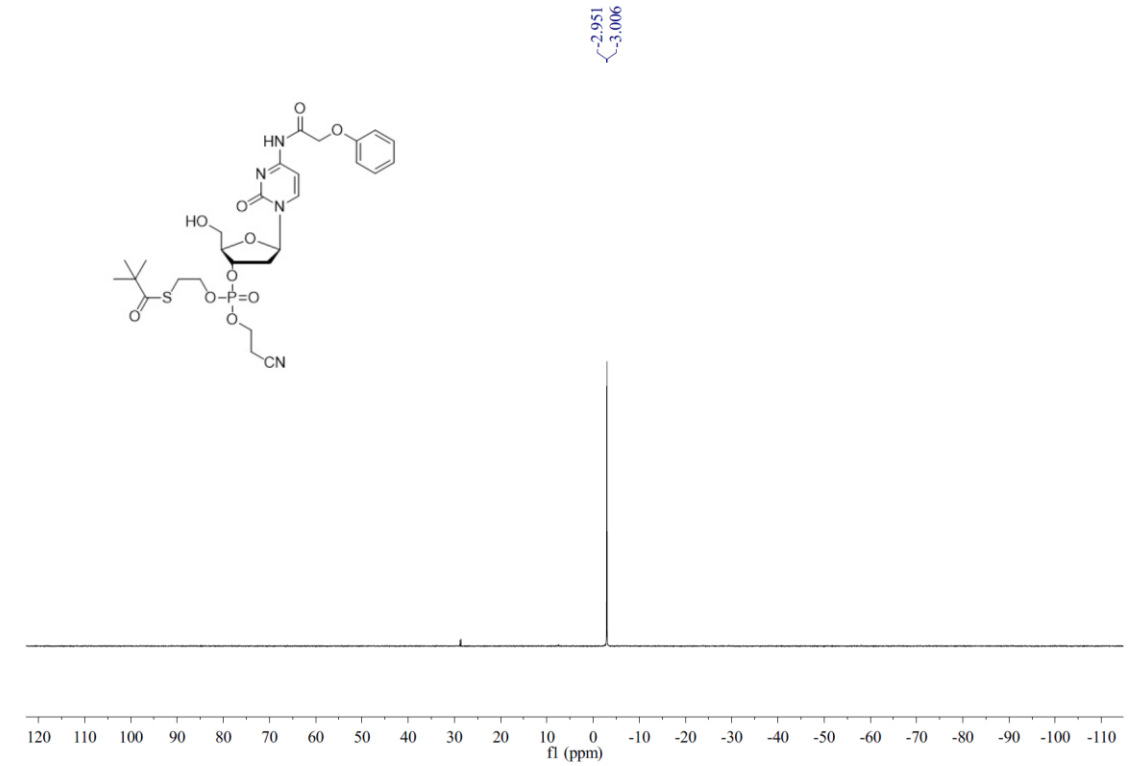




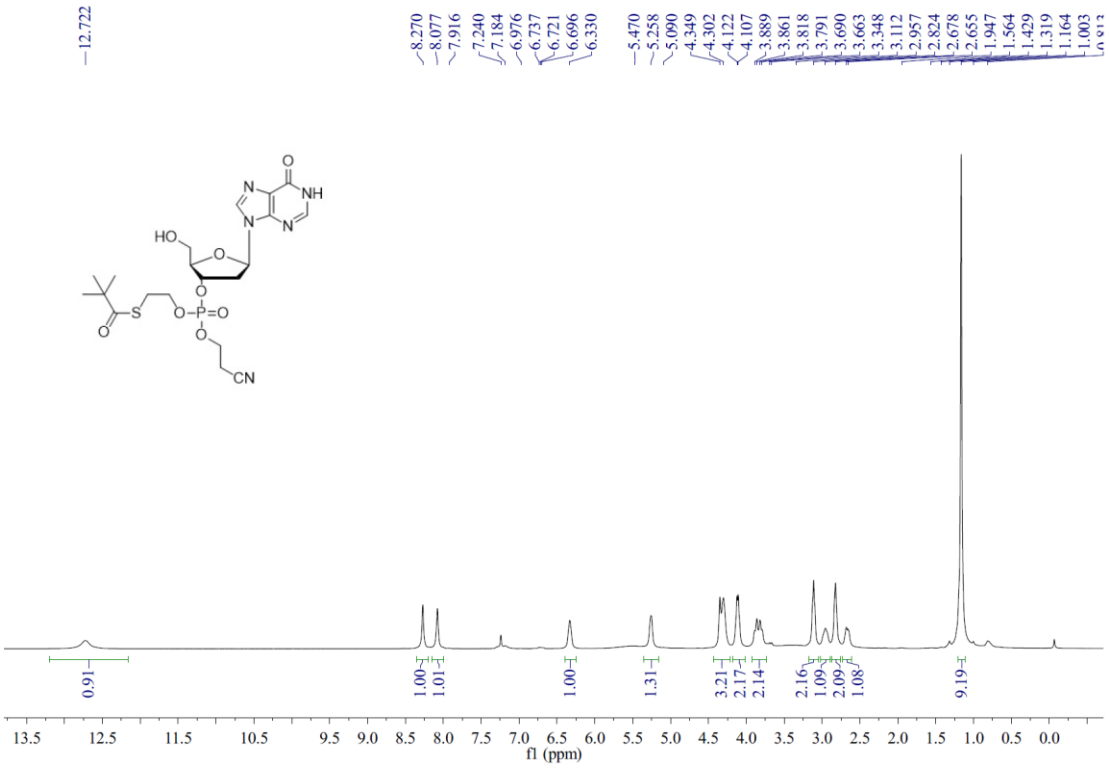
^{1}H, ^{13}C, ^{31}P NMR of **11c:**

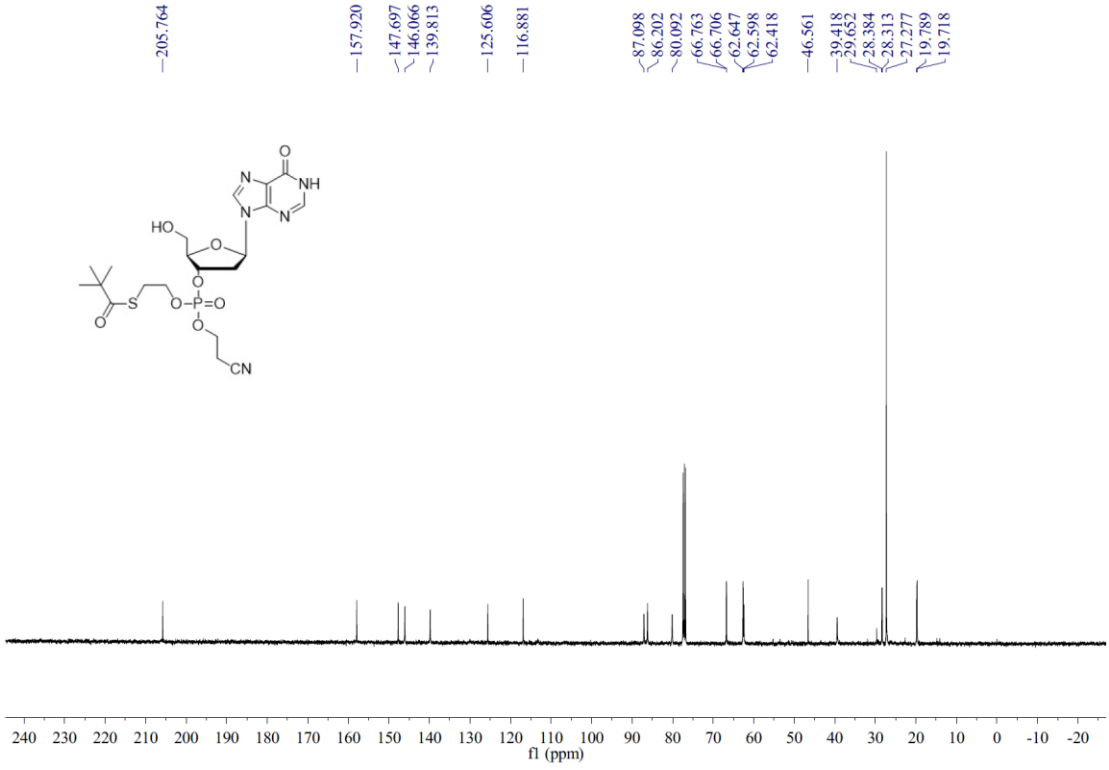


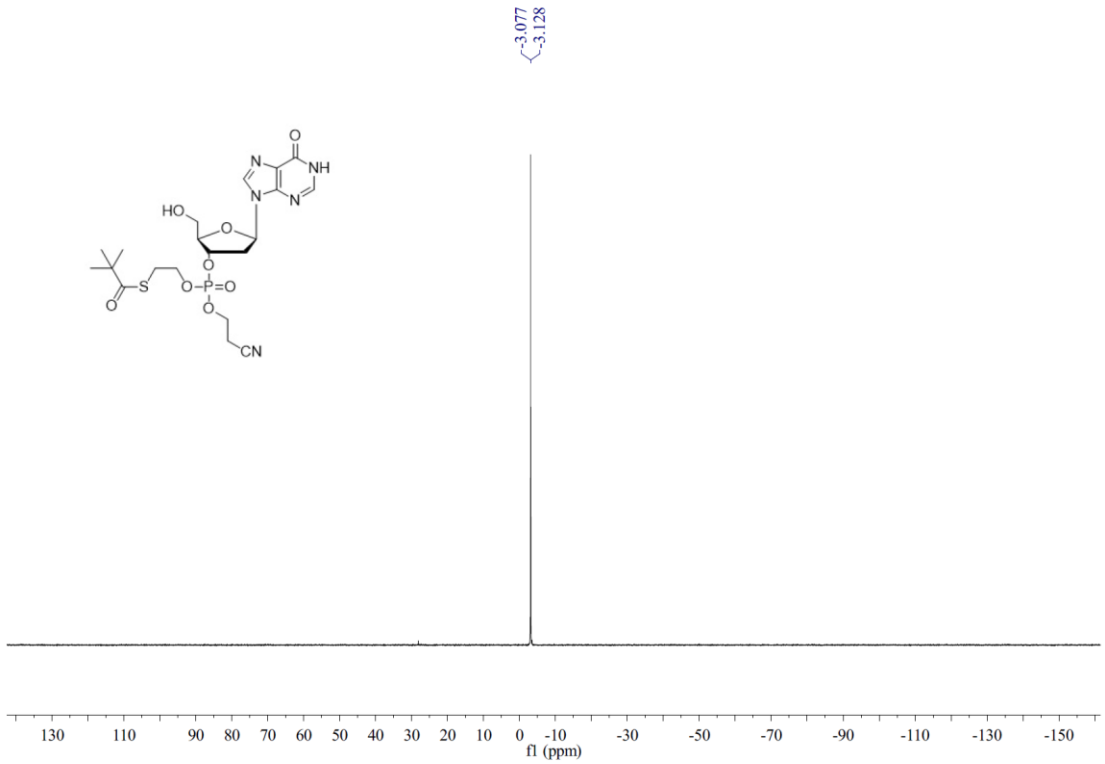

205.752
168.636
161.623
156.781
155.306
145.734
129.844
122.436
116.584
114.629
96.635
87.711
86.577
86.514
86.457
78.473
67.307
66.824
62.467
62.416
61.419
46.574
39.966
39.925
28.328
28.255
27.274
19.694
19.620
HN
N
O
HO
O
S
O-P=O
CN
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)


2.951
3.006
HN
N
O
HO
O
S
O-P=O
CN
120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20 -30 -40 -50 -60 -70 -80 -90 -100 -110
f1 (ppm)

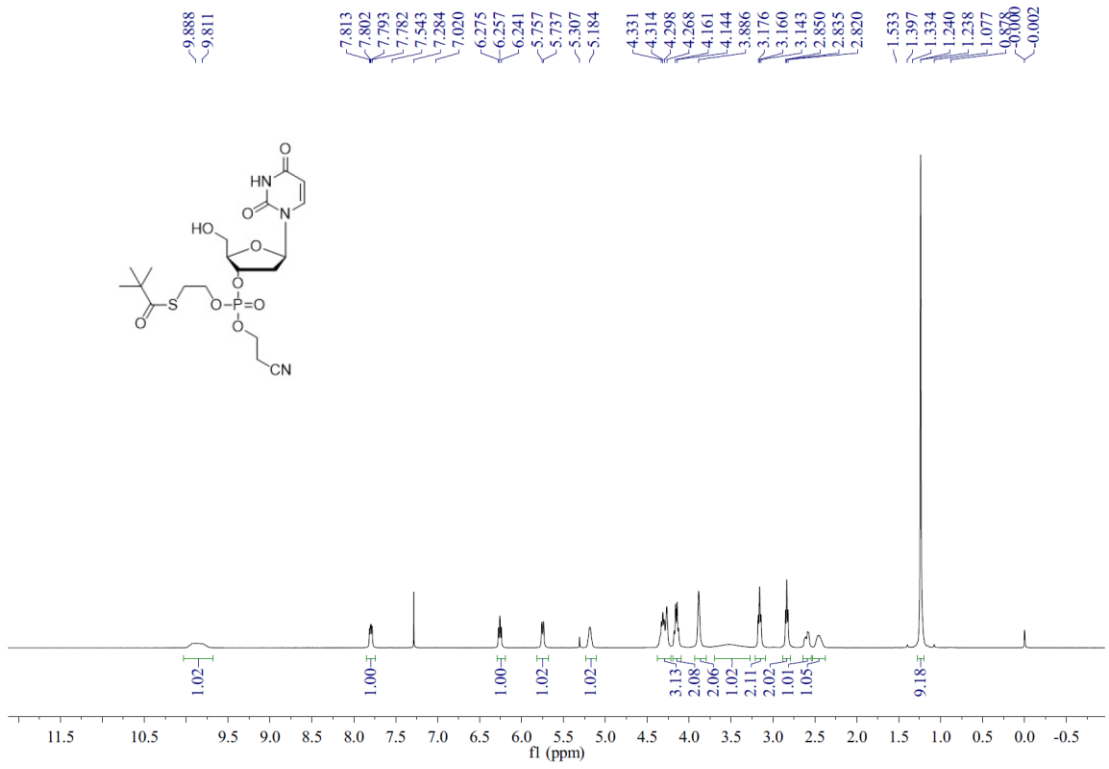
^{1}H, ^{13}C, ^{31}P NMR of **11d:**

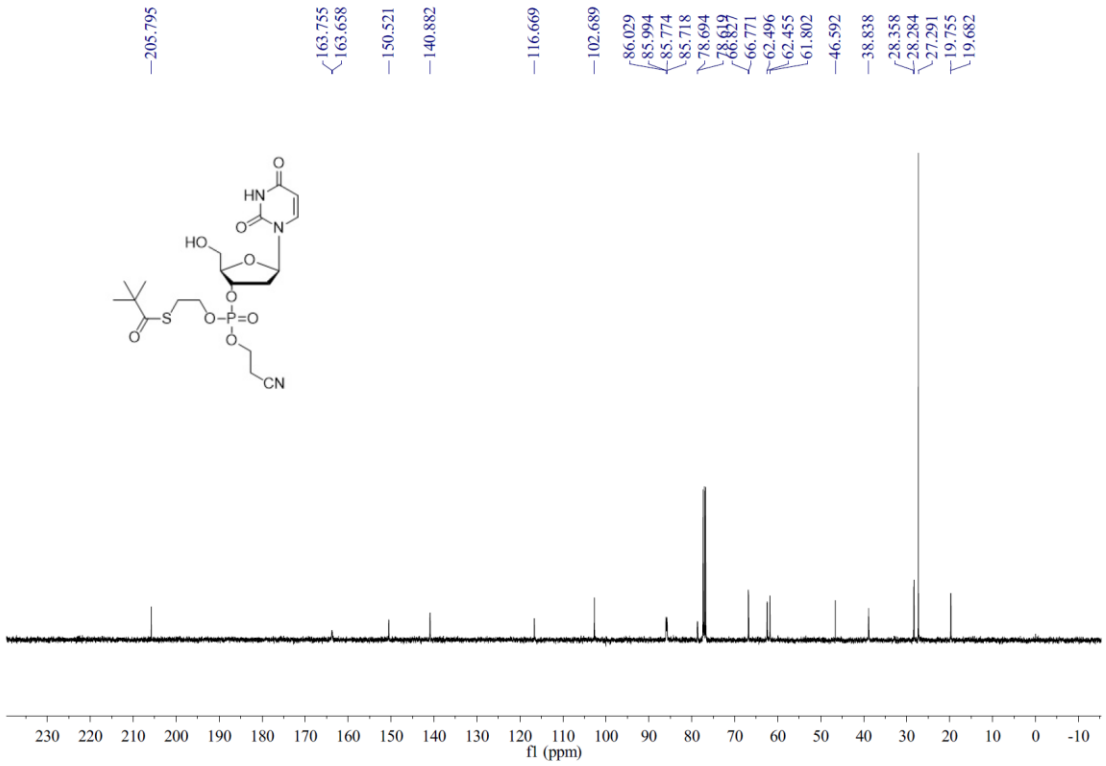


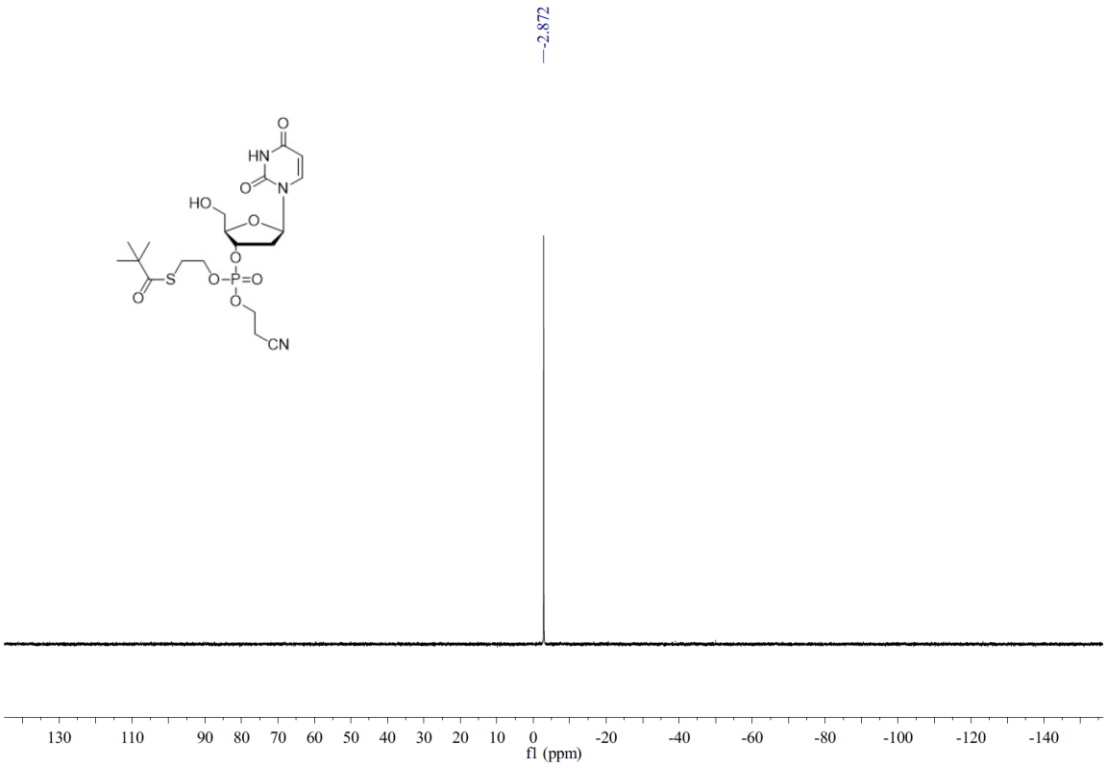




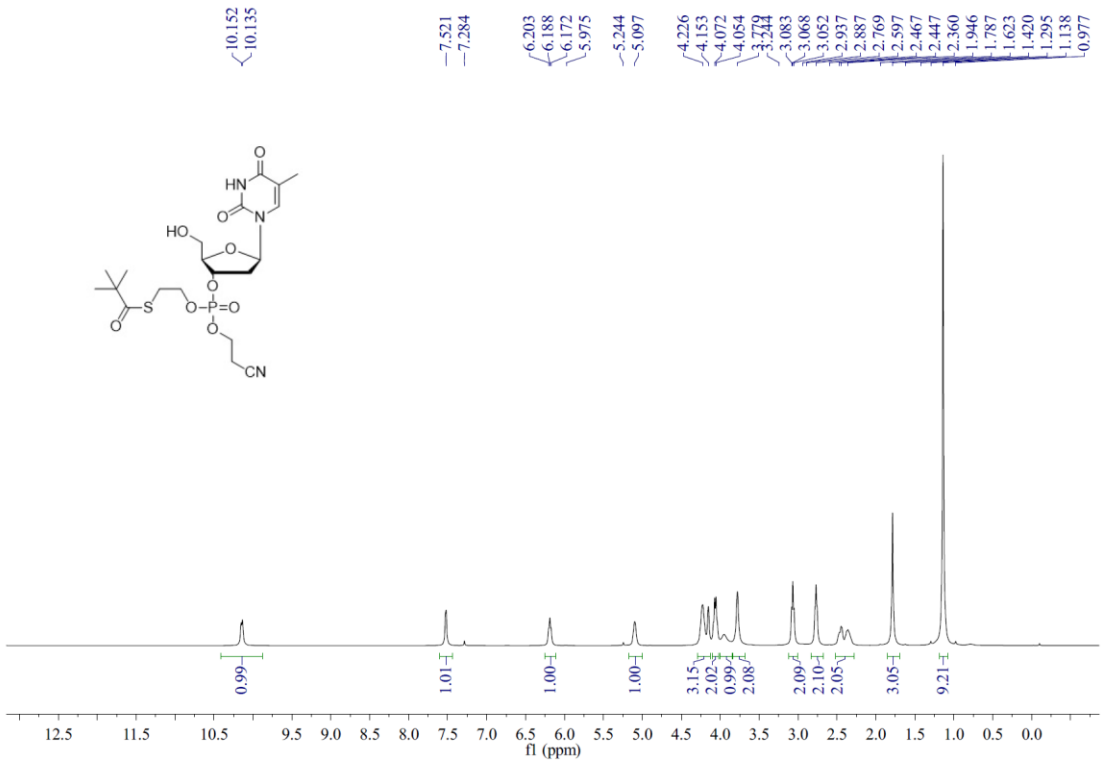
^{1}H, ^{13}C, ^{31}P NMR of **11e:**

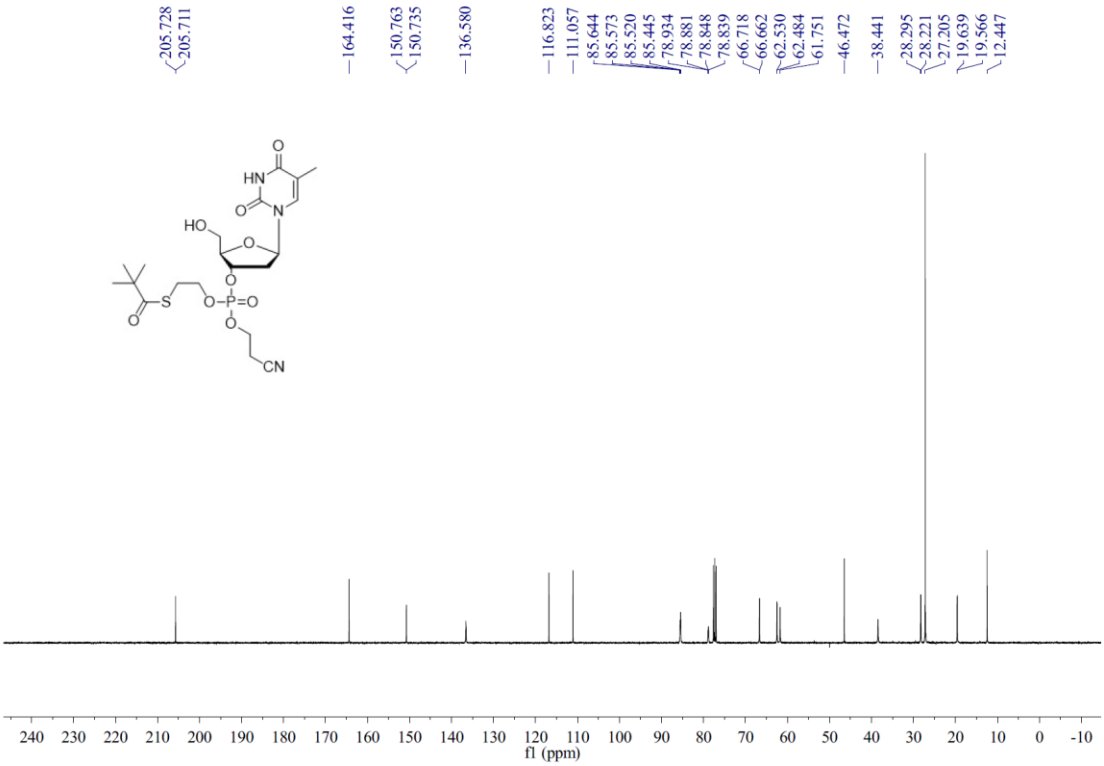


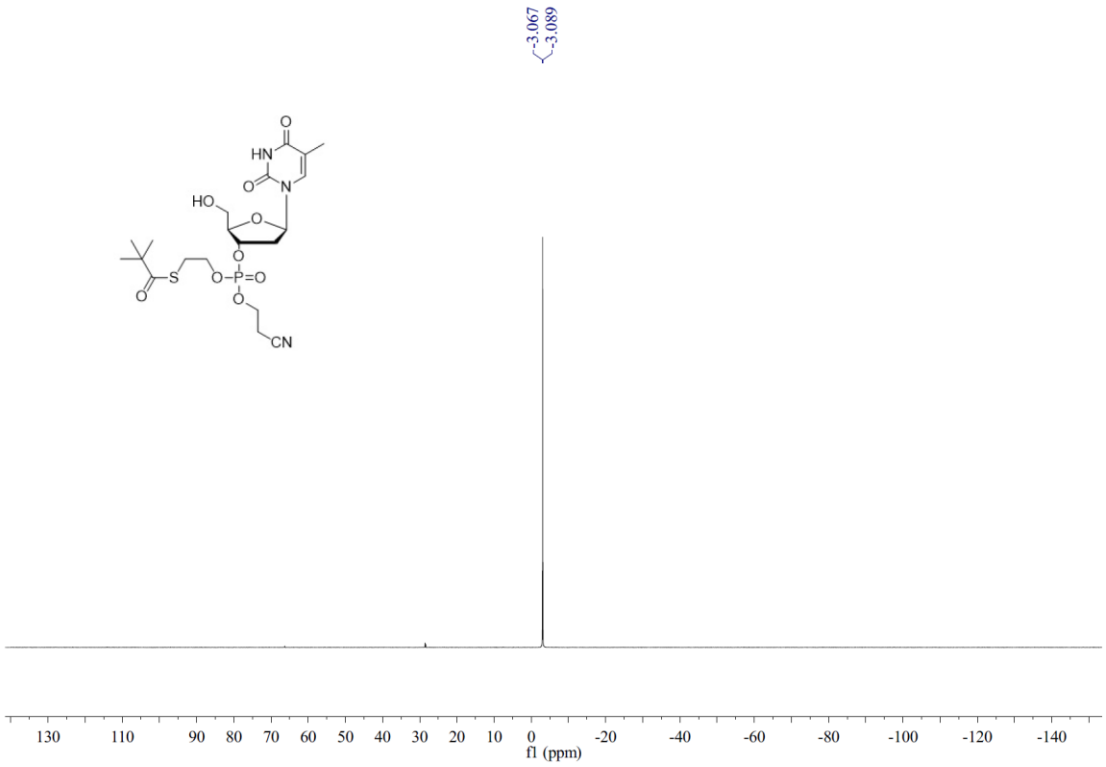

205.795
163.755
163.658
150.521
140.882
116.669
102.689
86.029
85.994
85.774
85.718
78.694
78.619
78.527
66.771
62.496
62.455
61.802
46.592
38.838
28.358
28.284
27.291
19.755
19.682
HO
HN
O
N
O
O
O
S
O-P=O
O
CN
230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)


2.872
HO
HN
O
N
O
O
O
S
O-P=O
O
CN
130 110 90 80 70 60 50 40 30 20 10 0 -20 -40 -60 -80 -100 -120 -140
f1 (ppm)

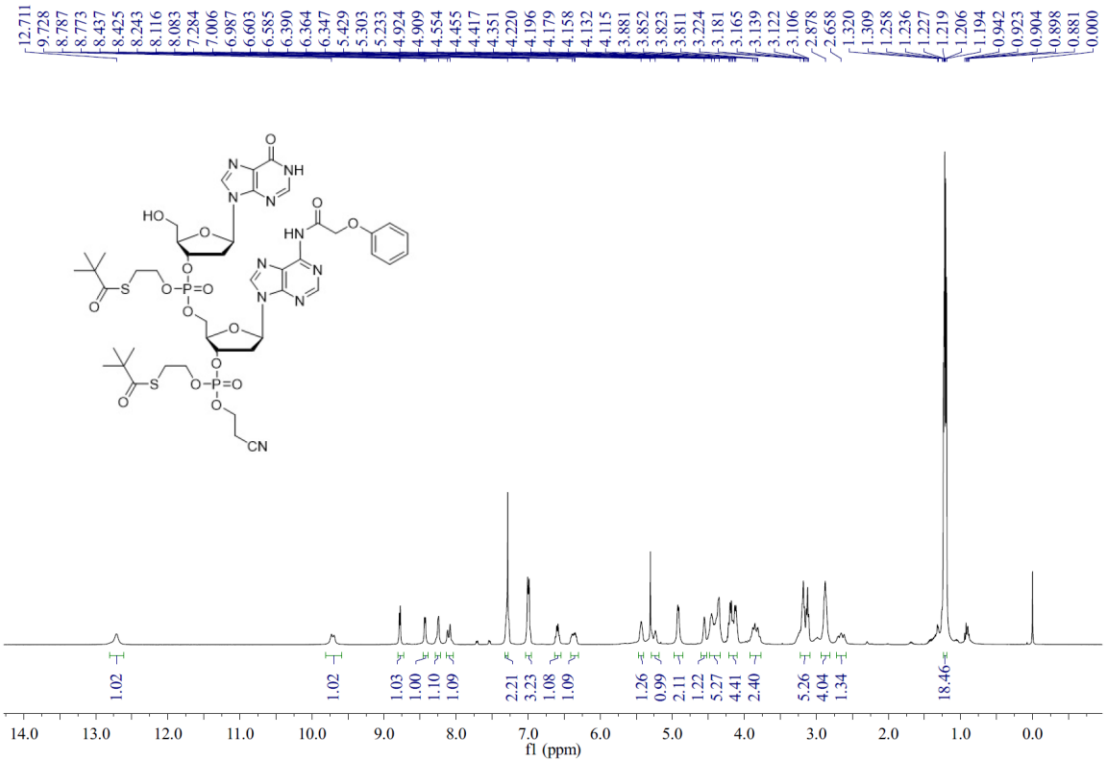
^{1}H, ^{13}C, ^{31}P NMR of **11f:**

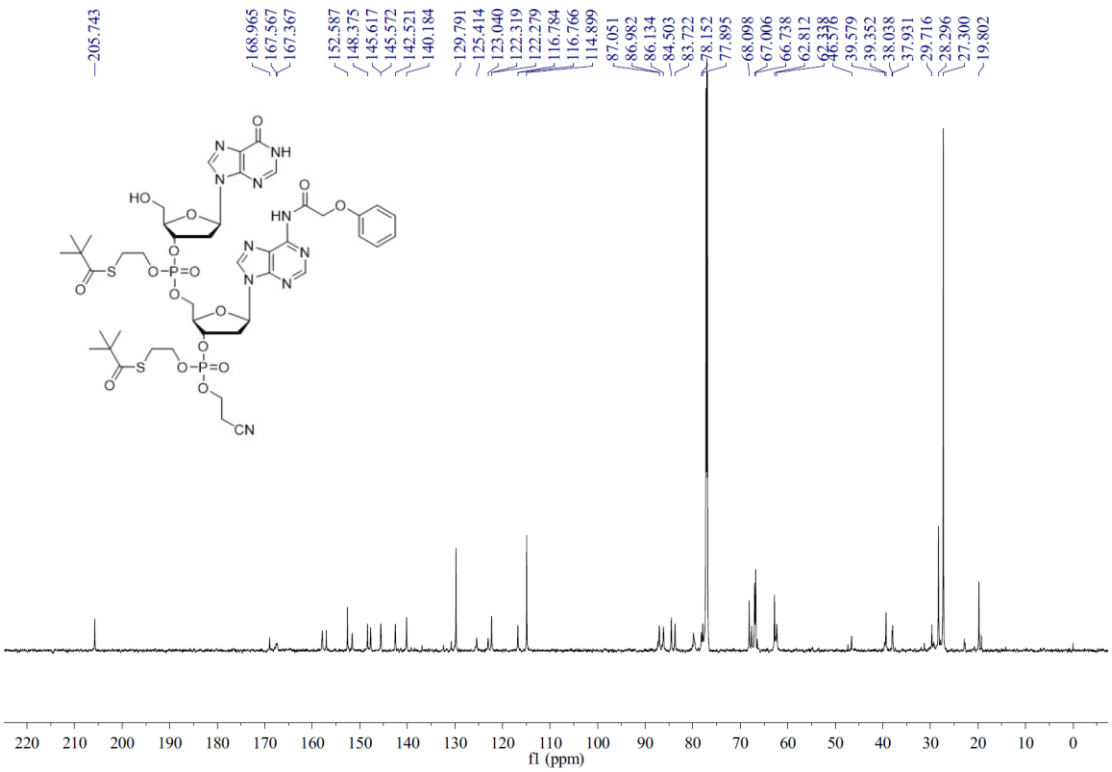


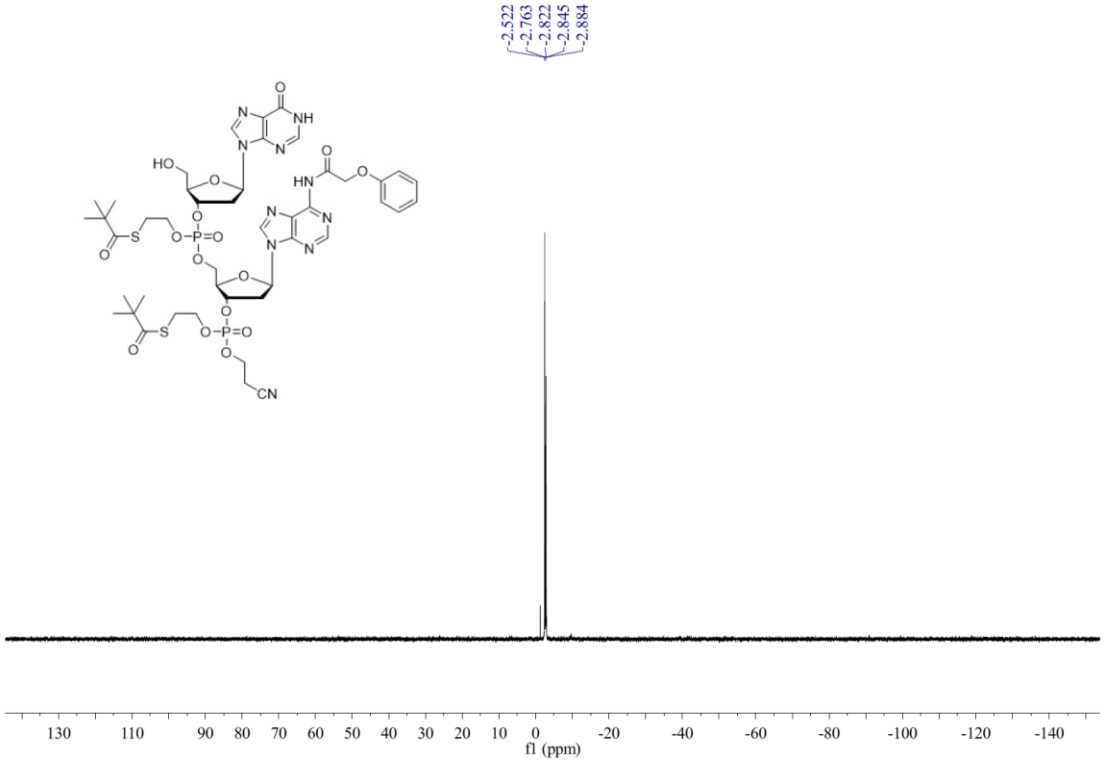




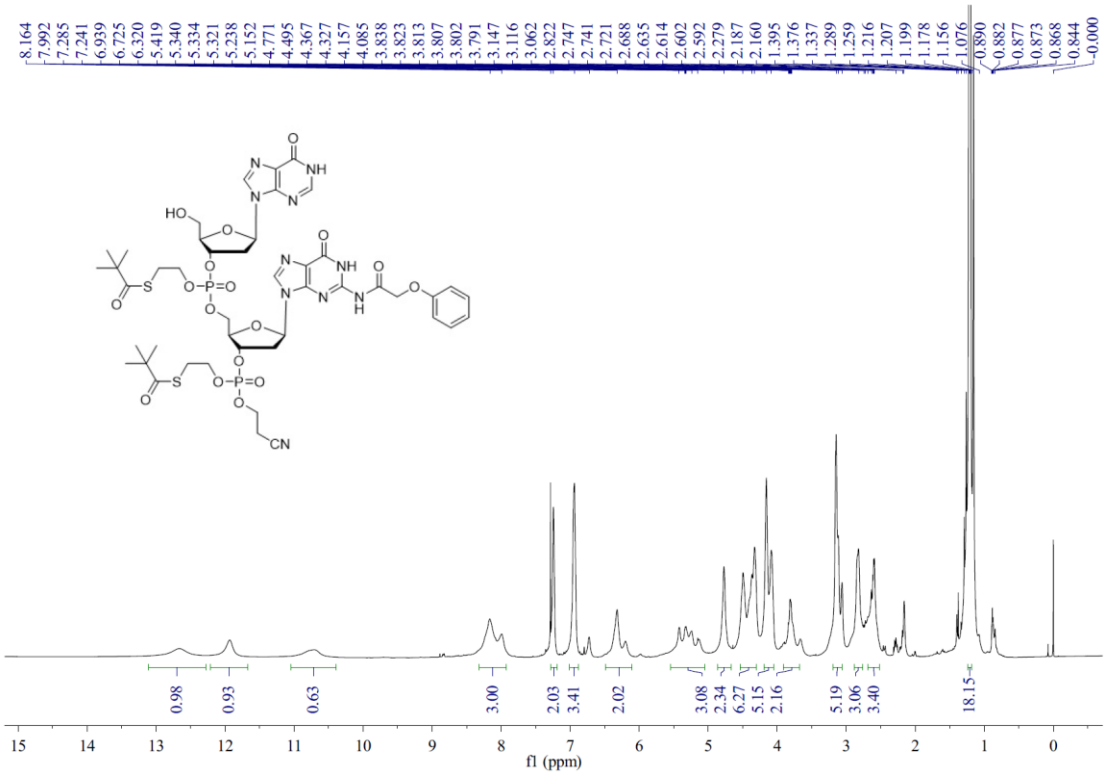
^{1}H, ^{13}C, ^{31}P NMR of **12a:**

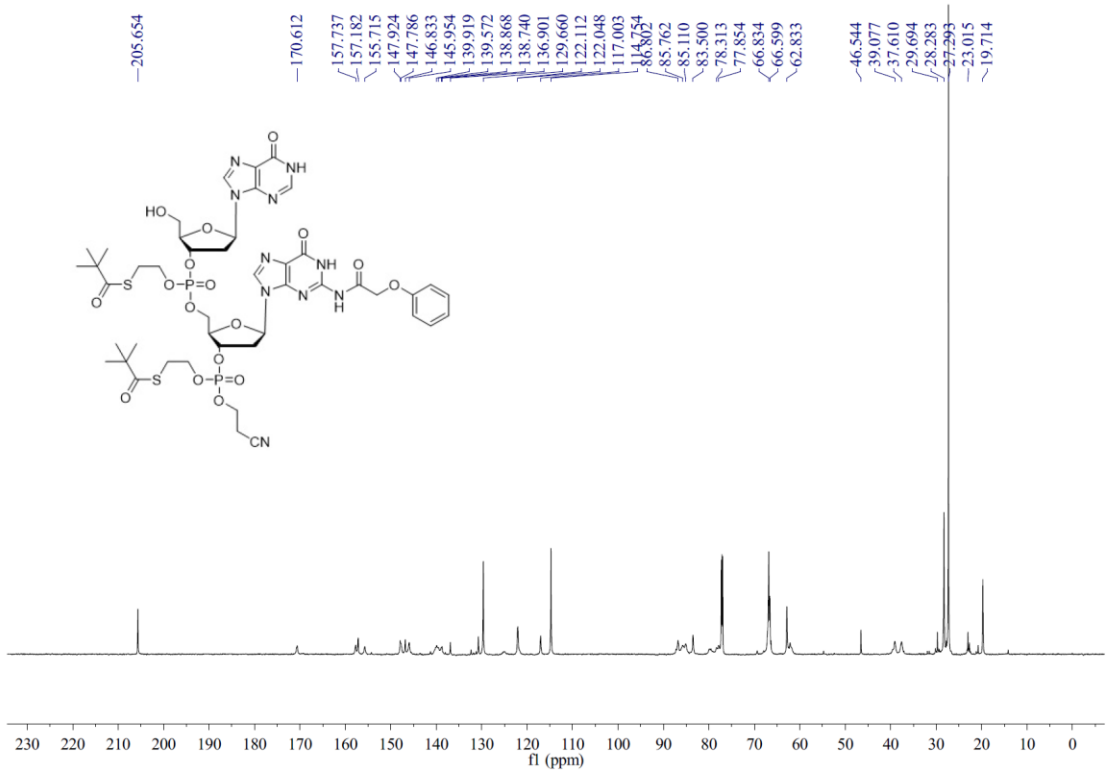


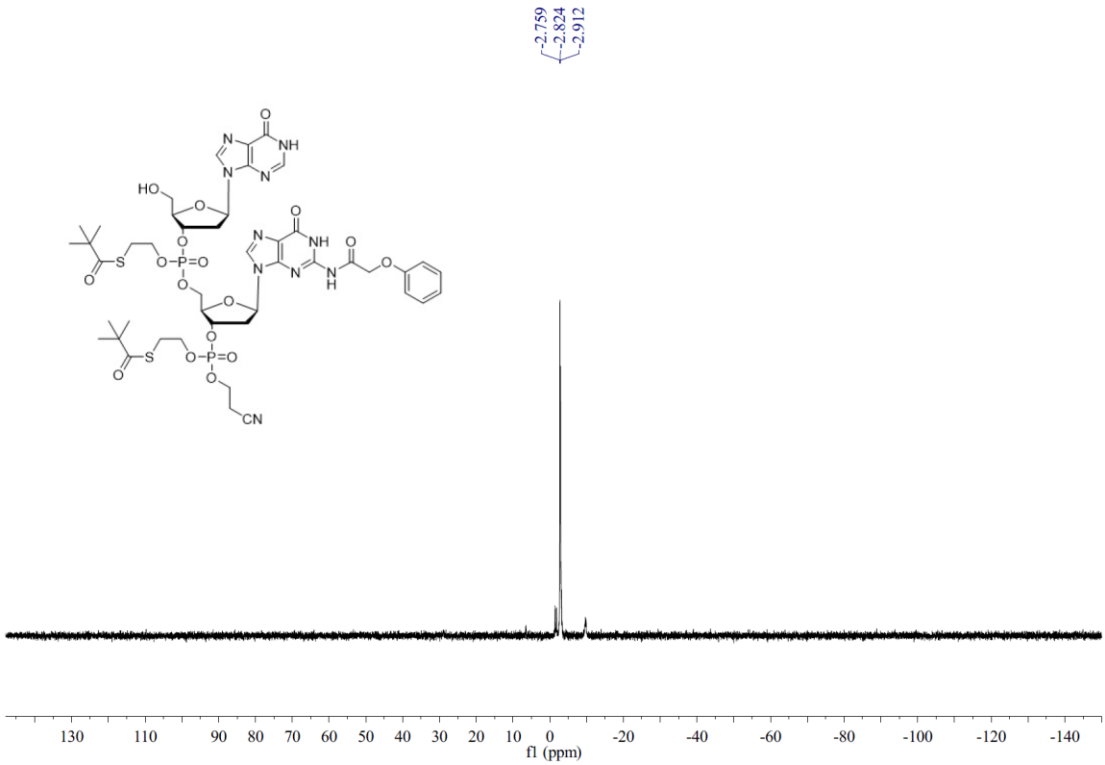

205.743
168.965
167.567
167.367
152.587
148.375
145.617
145.572
142.521
140.184
129.791
125.414
123.040
122.319
122.279
116.784
116.766
114.899
87.051
86.982
86.134
84.503
83.722
78.152
77.895
68.098
67.006
66.738
62.812
62.338
46.578
39.579
39.352
38.038
37.931
29.716
28.296
27.300
19.802
HO
NH
HN
O
N
S
O-P=O
CN
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)


2.522
2.763
2.822
2.845
2.884
HO
NH
HN
O
N
S
O-P=O
CN
130 110 90 80 70 60 50 40 30 20 10 0 -20 -40 -60 -80 -100 -120 -140
f1 (ppm)

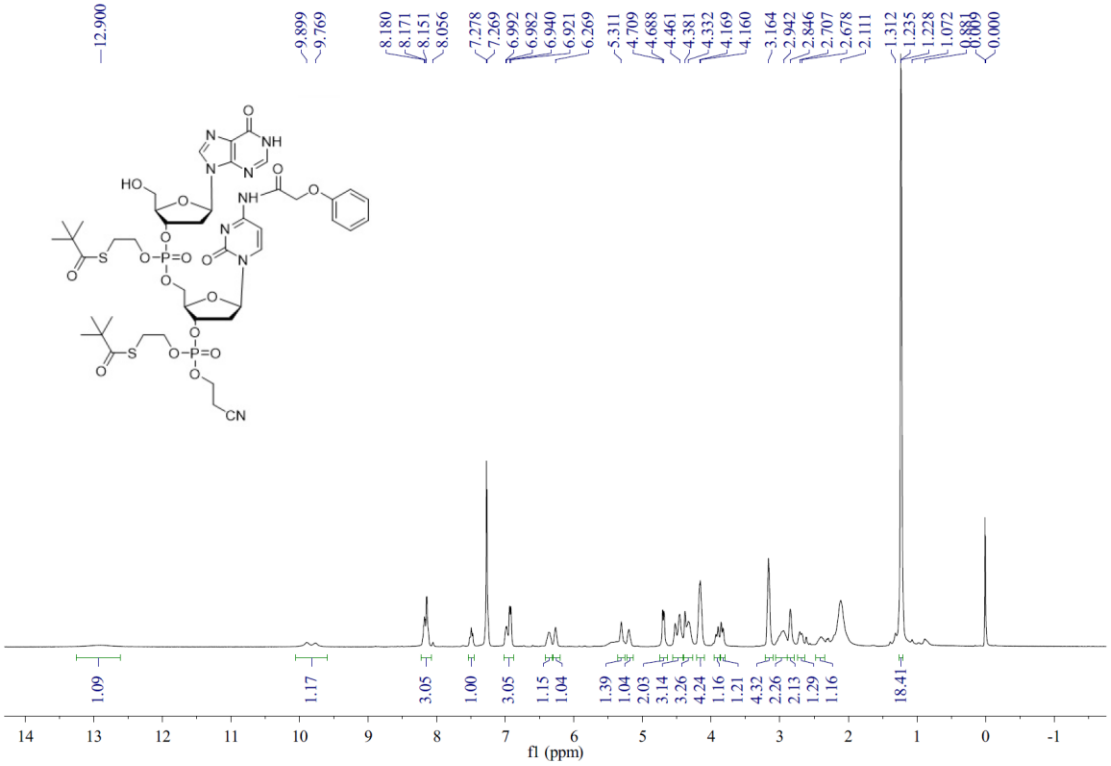
^{1}H, ^{13}C, ^{31}P NMR of **12b:**

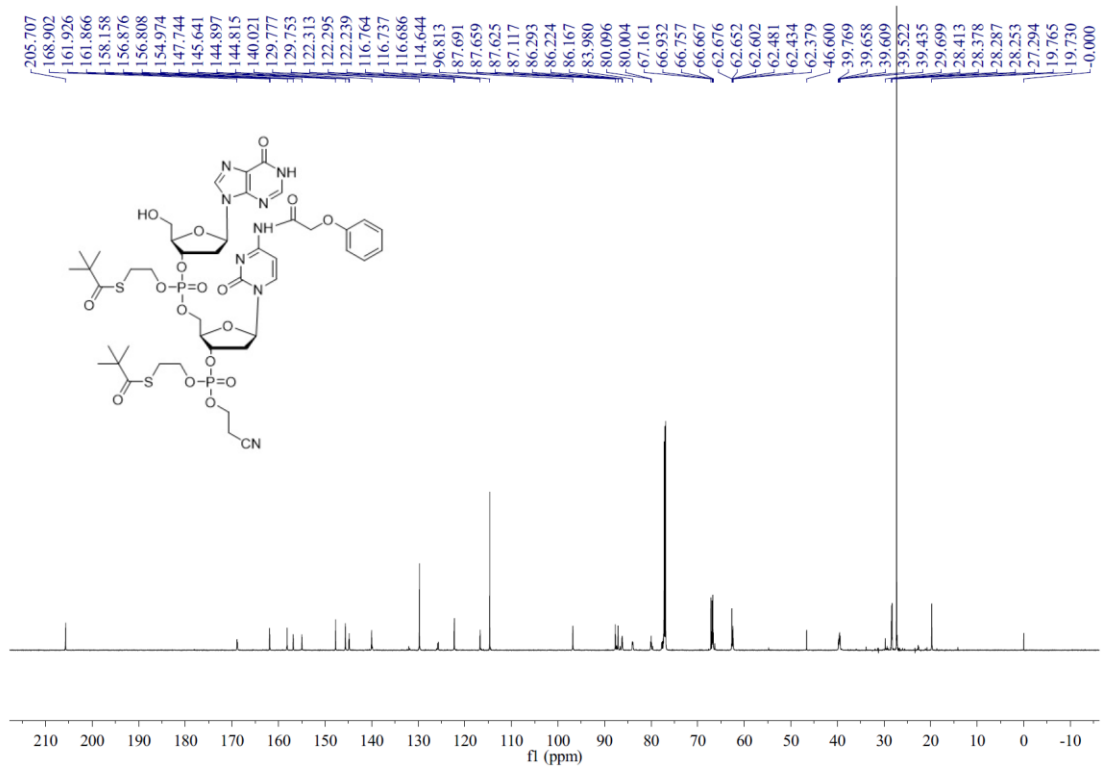


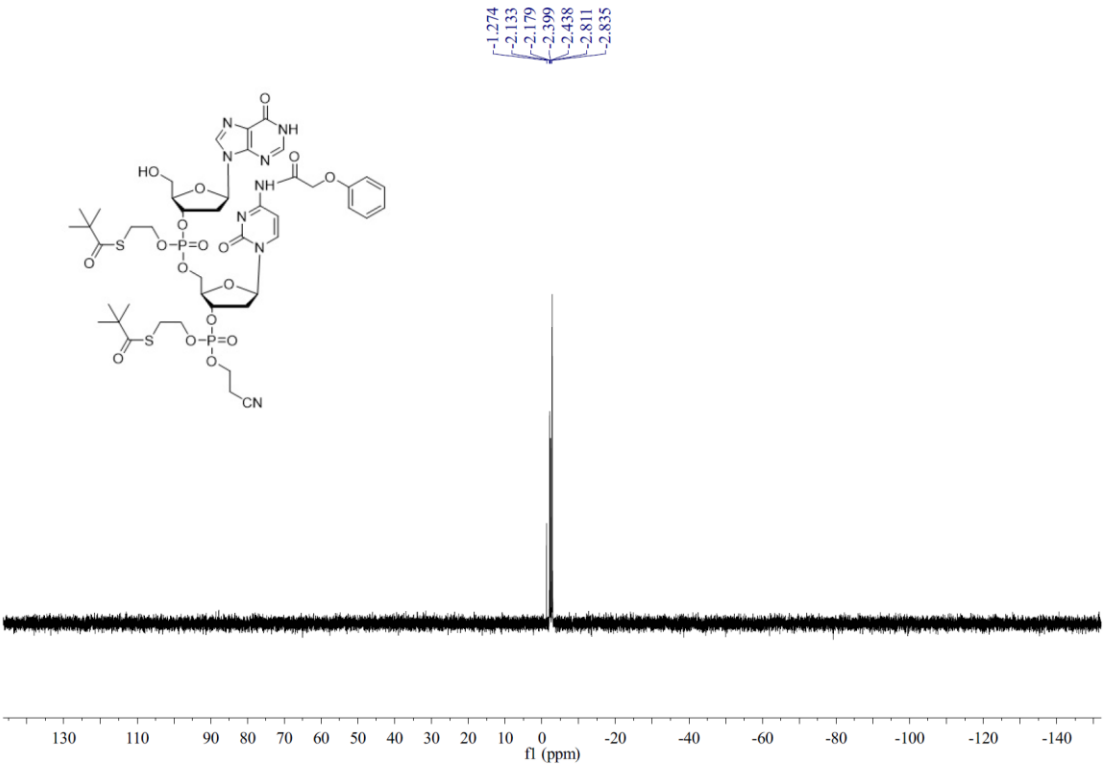




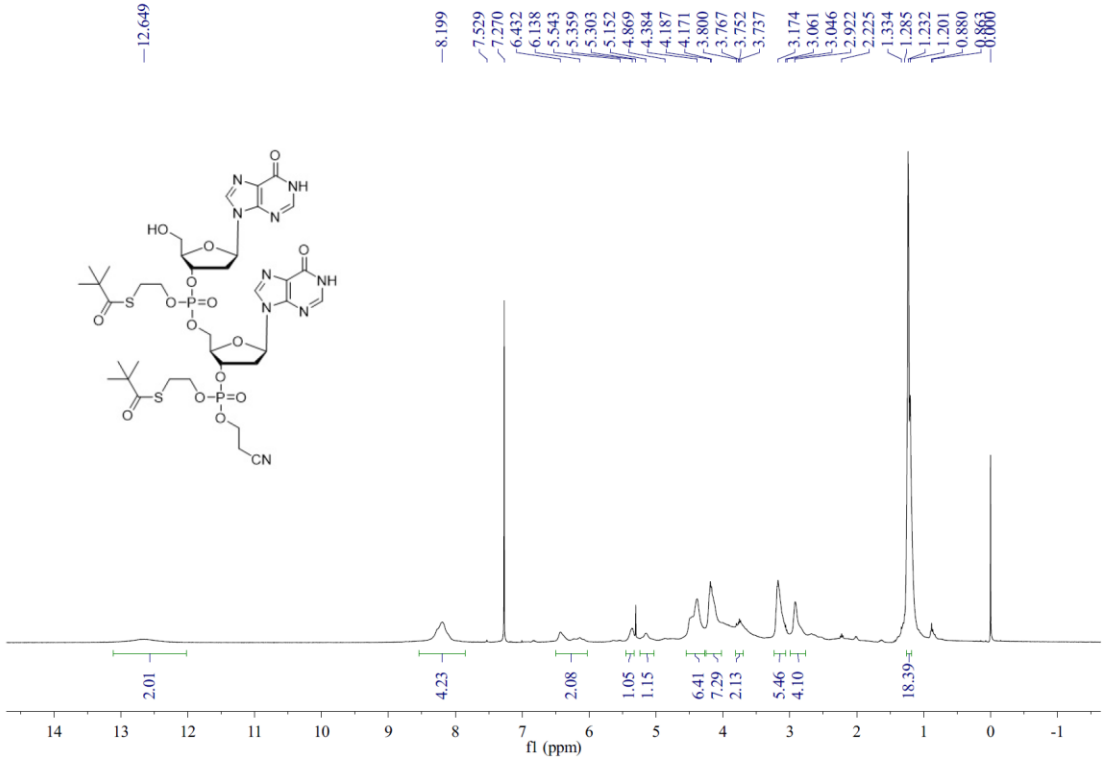
^{1}H, ^{13}C, ^{31}P NMR of **12c:**

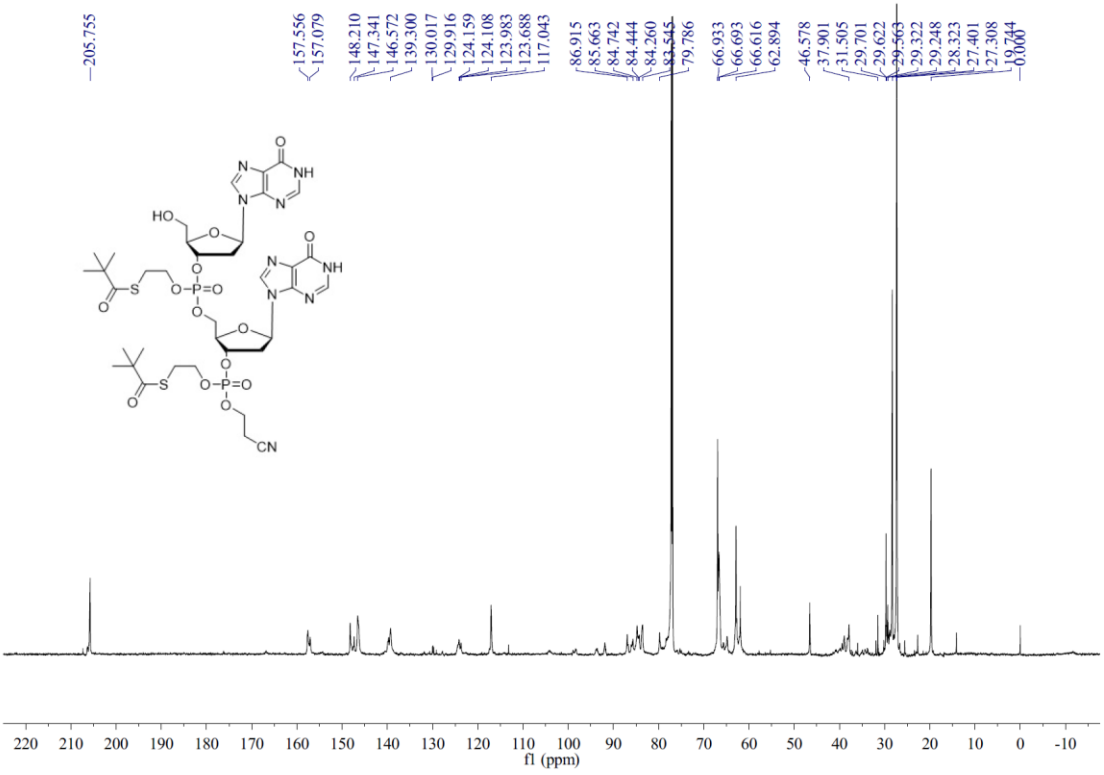


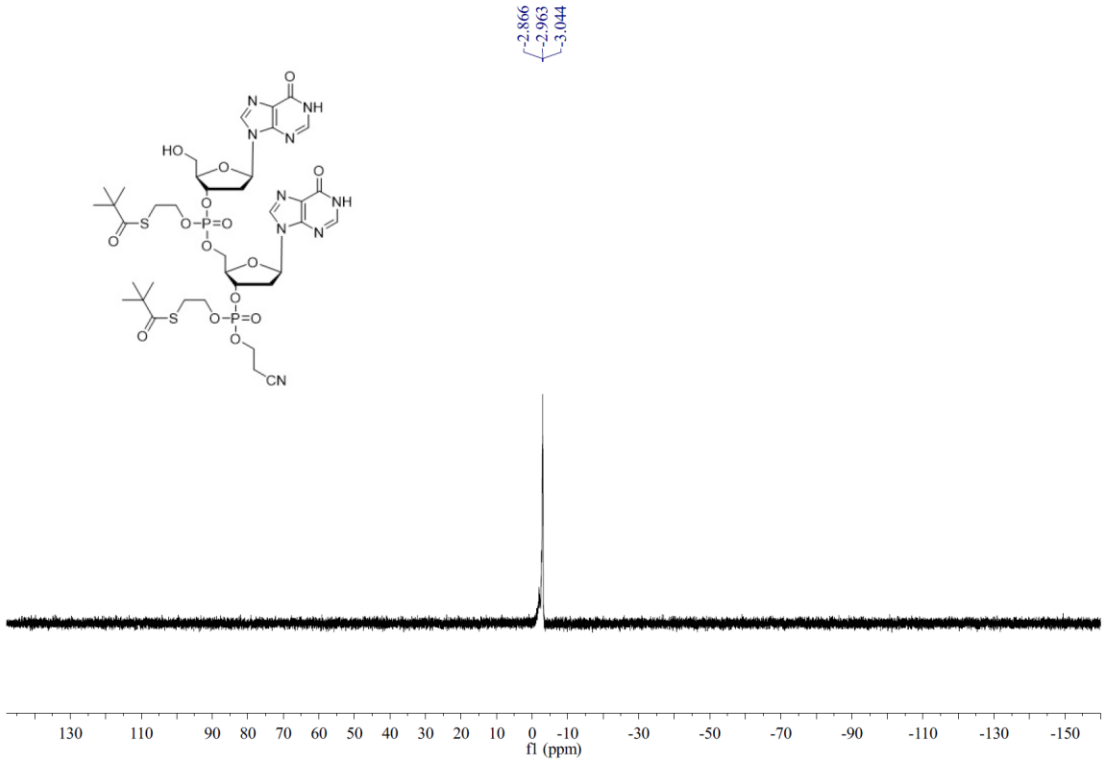

205.707
168.902
161.926
161.866
158.158
156.876
156.808
154.974
147.744
145.641
144.897
144.815
140.021
129.777
129.753
122.313
122.295
122.239
116.764
116.737
116.686
114.644
96.813
87.691
87.659
87.625
87.117
86.293
86.224
86.167
83.980
80.096
80.004
67.161
66.932
66.757
66.667
62.676
62.652
62.602
62.481
62.434
62.379
46.600
39.769
39.658
39.609
39.522
39.435
29.699
28.413
28.378
28.287
28.253
27.294
19.765
19.730
-0.000
HO
NH
O
N
S
P
CN
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)


-1.274
-2.133
-2.179
-2.399
-2.438
-2.811
-2.835
HO
NH
O
N
S
P
CN
130 110 90 80 70 60 50 40 30 20 10 0 -20 -40 -60 -80 -100 -120 -140
f1 (ppm)

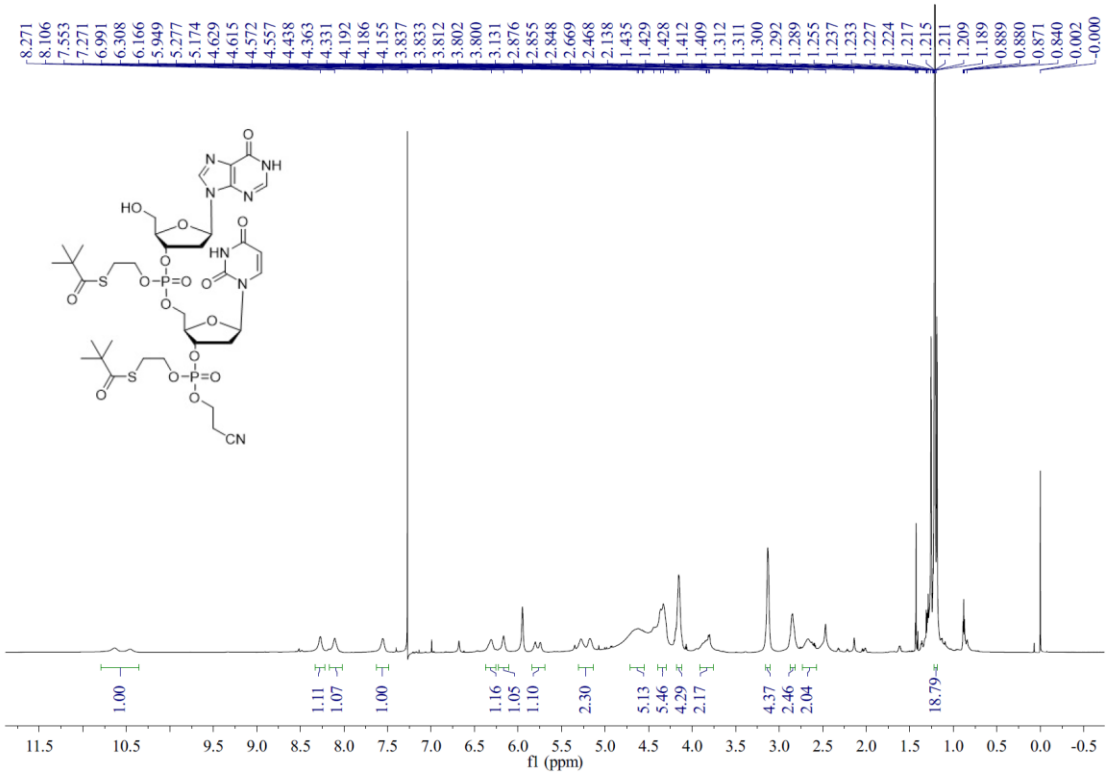
^{1}H, ^{13}C, ^{31}P NMR of **12d:**

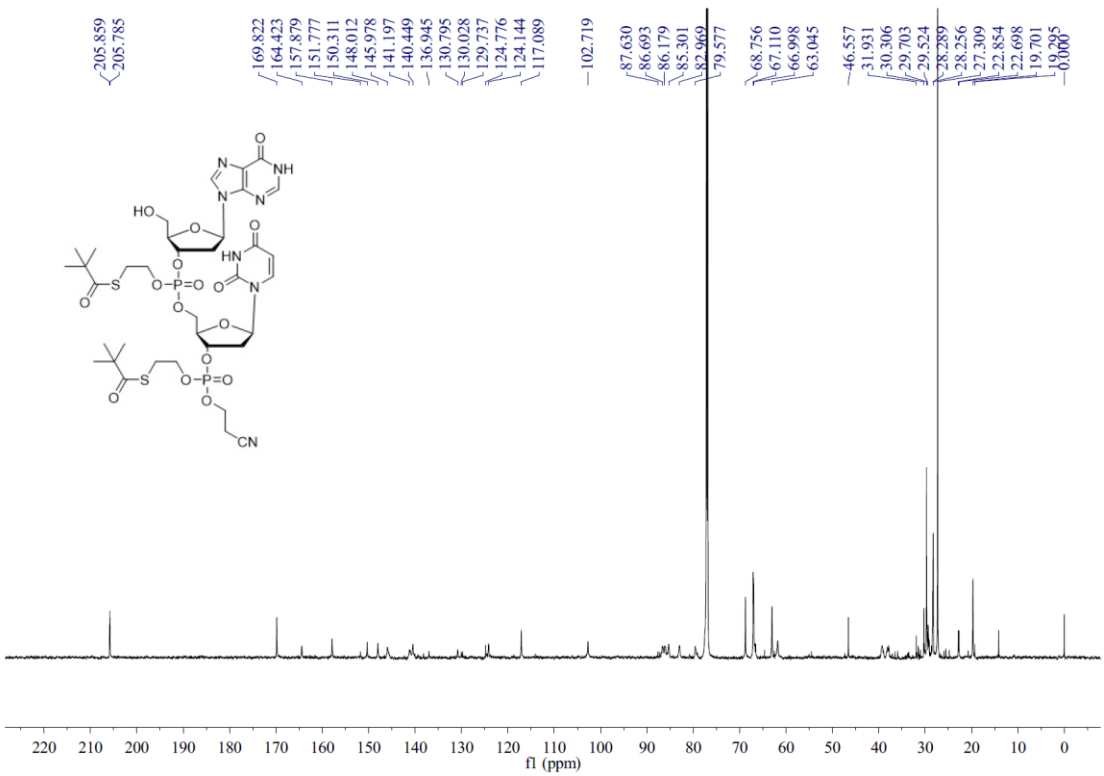


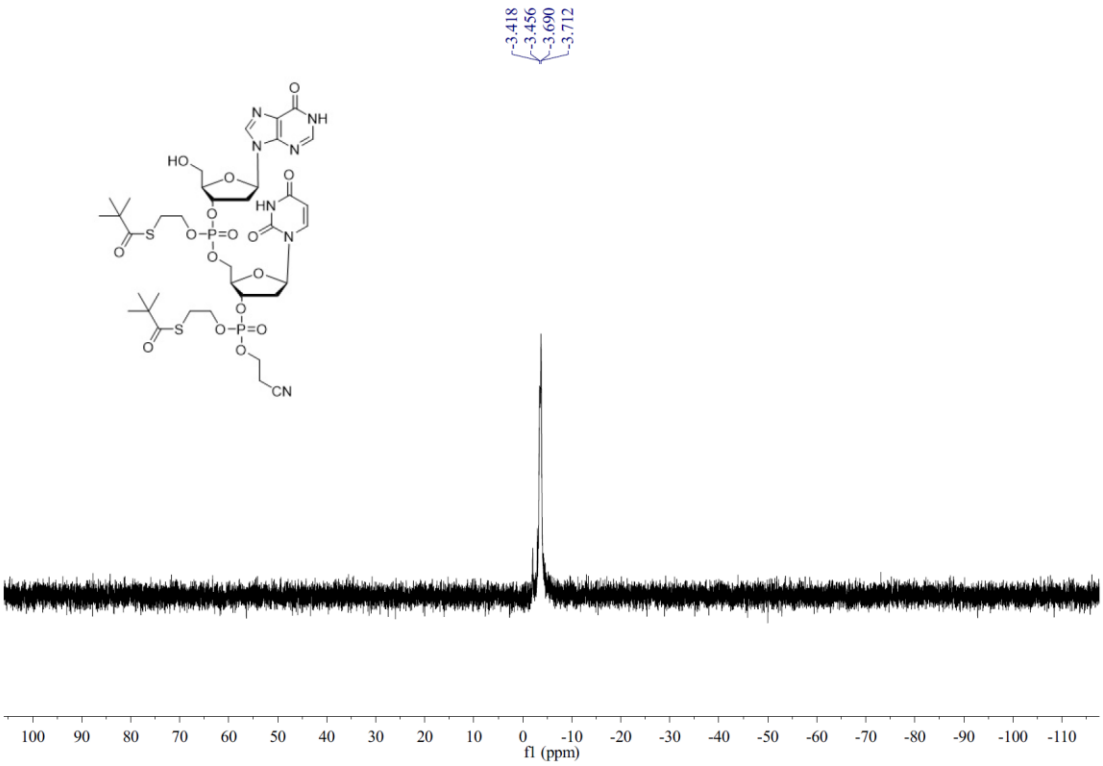




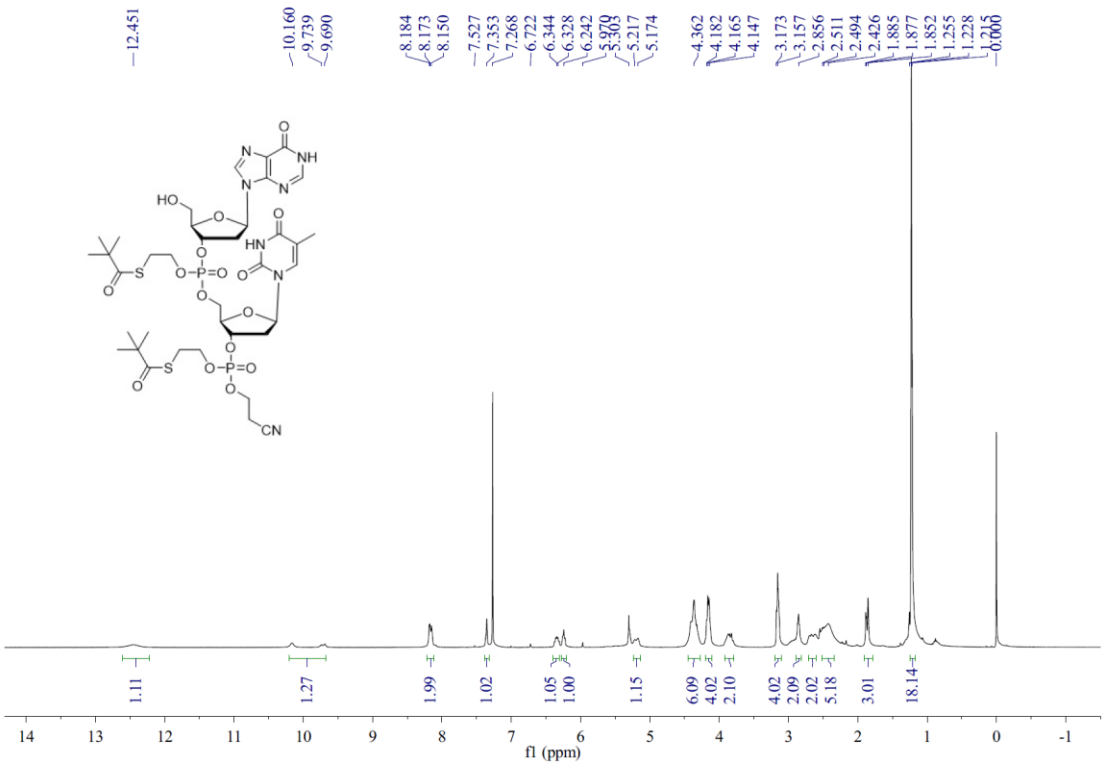
^{1}H, ^{13}C, ^{31}P NMR of **12e:**

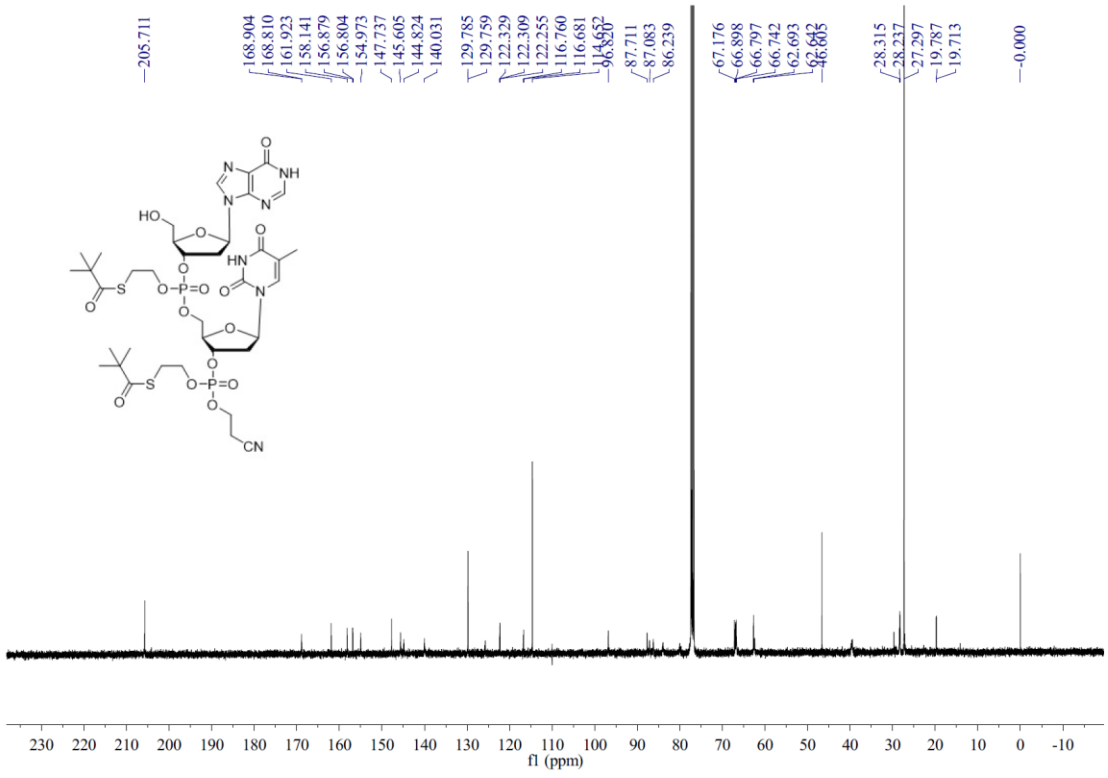


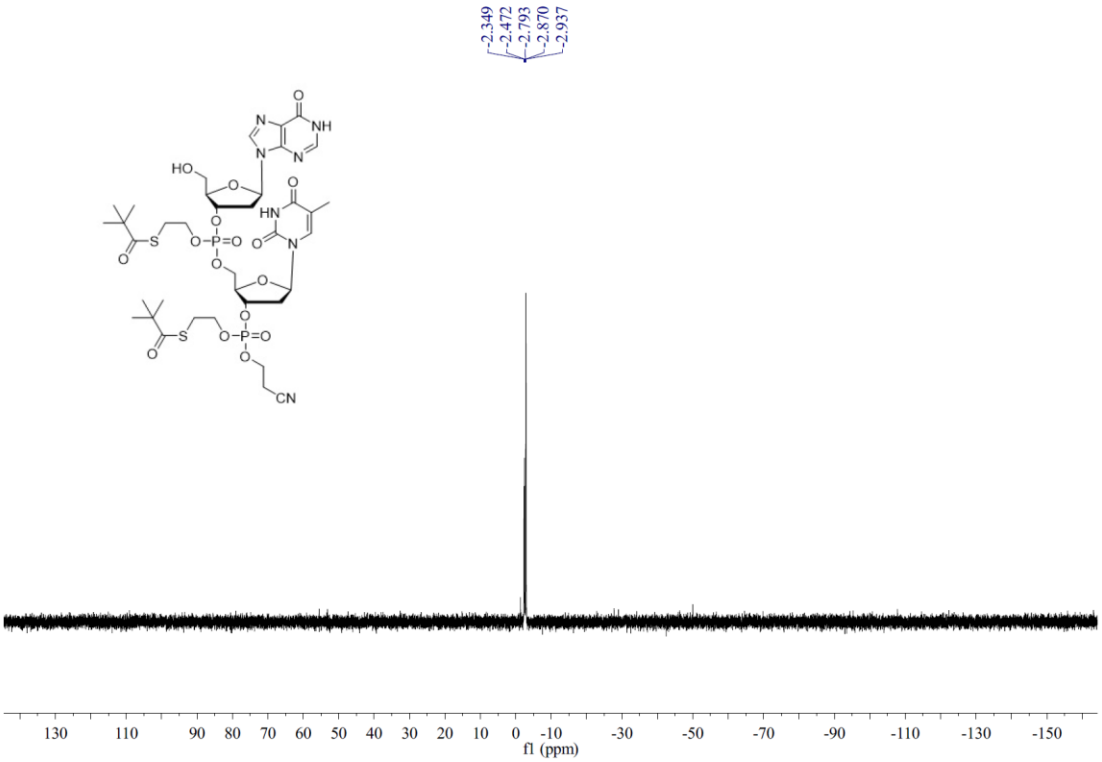

205.859
205.785
169.822
164.423
157.879
151.777
150.311
148.012
145.978
141.197
140.449
136.945
130.795
130.028
129.737
124.776
124.144
117.089
102.719
87.630
86.693
86.179
85.301
82.969
79.577
68.756
67.110
66.998
63.045
46.557
31.931
30.306
29.703
29.524
28.289
28.256
27.309
22.854
22.698
19.701
19.295
0.000
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)


3.418
3.456
3.690
3.712
100 90 80 70 60 50 40 30 20 10 0 -10 -20 -30 -40 -50 -60 -70 -80 -90 -100 -110
f1 (ppm)

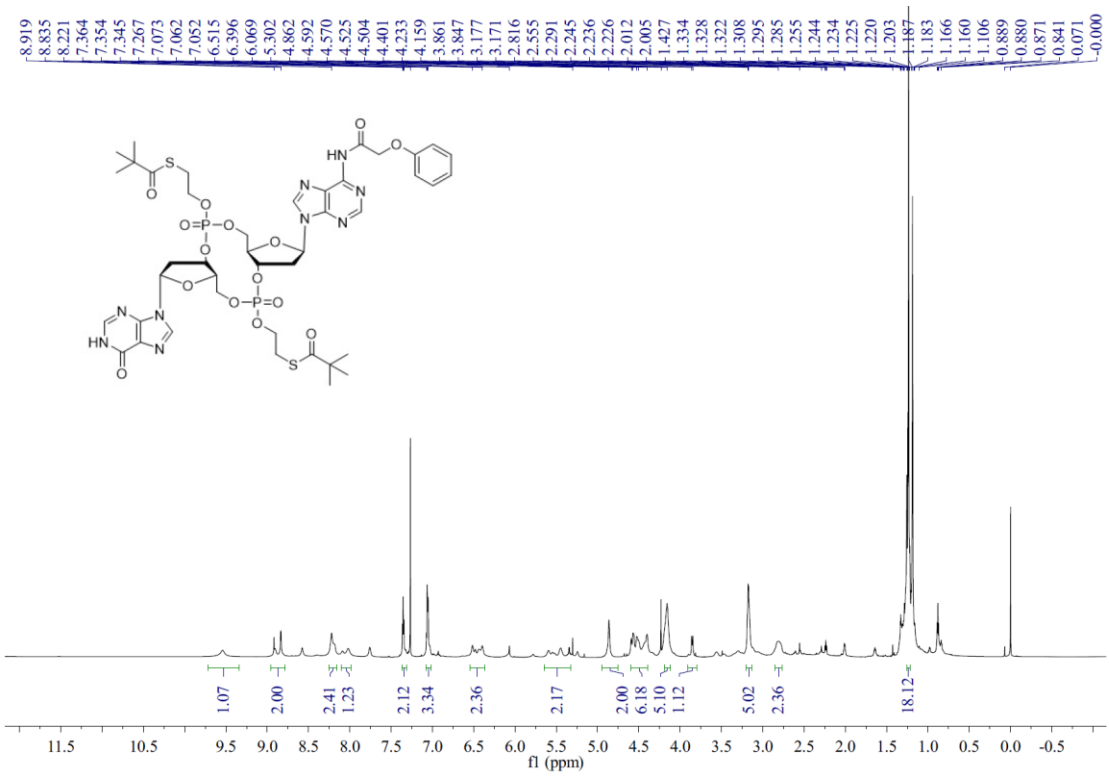
^{1}H, ^{13}C, ^{31}P NMR of **12f:**

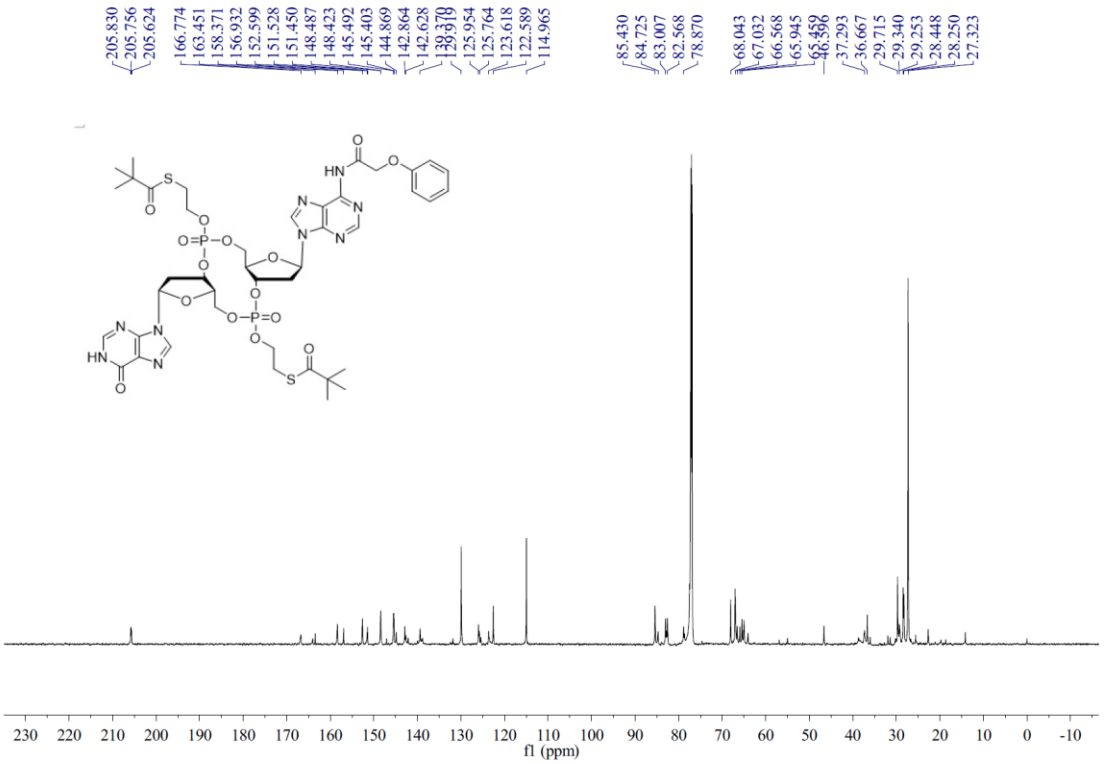


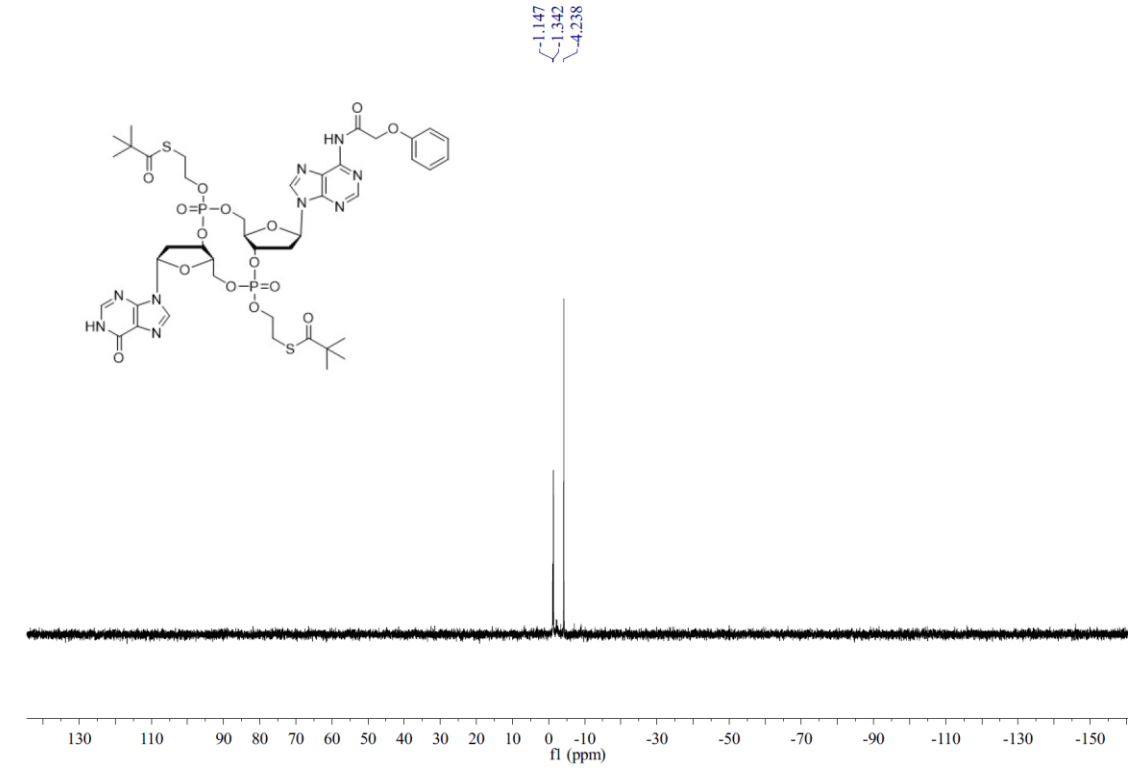




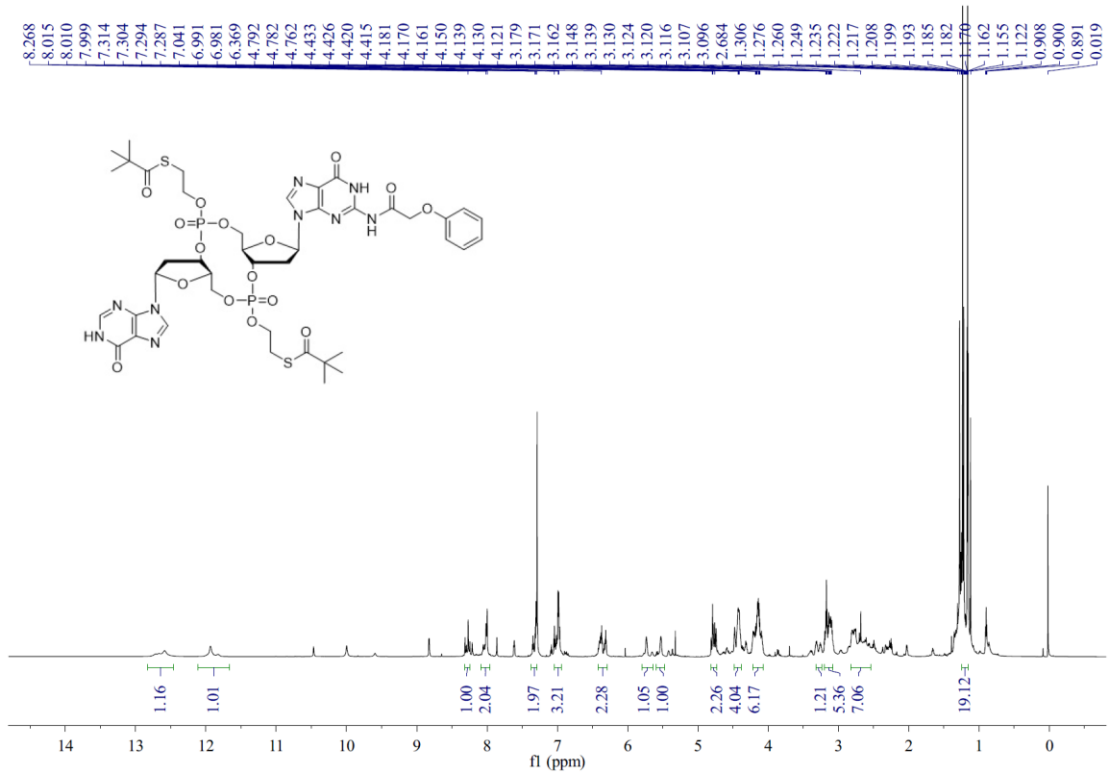
^{1}H, ^{13}C, ^{31}P NMR of **13a:**

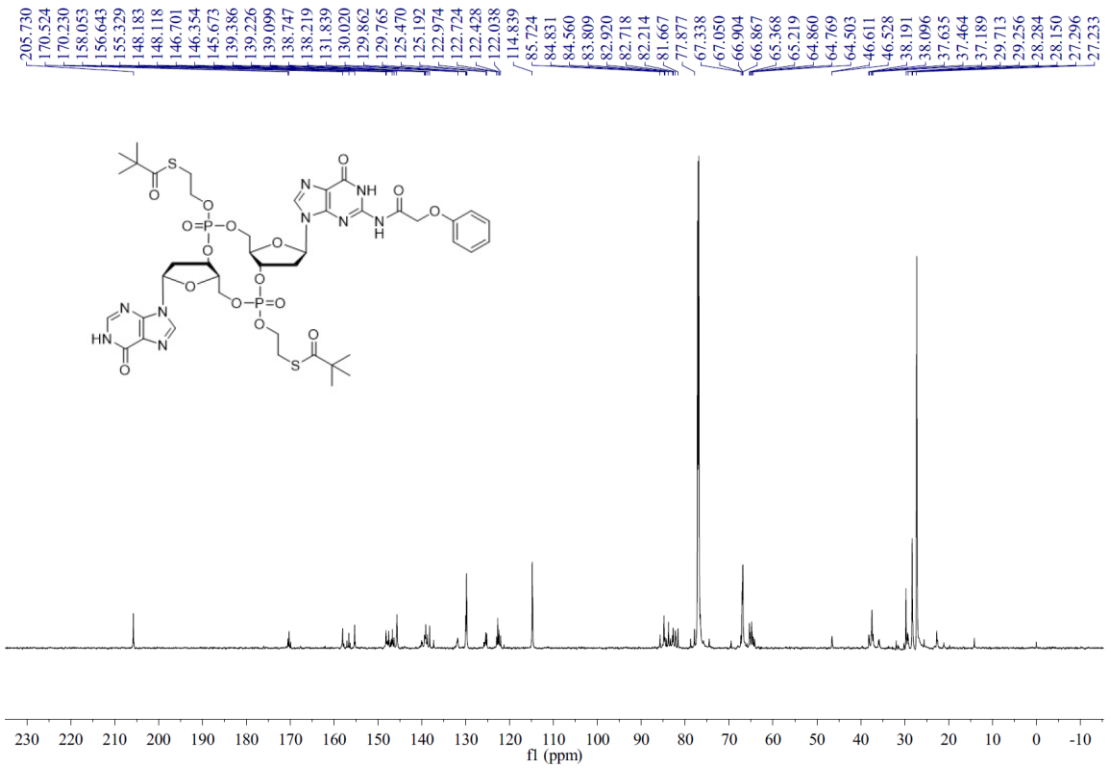


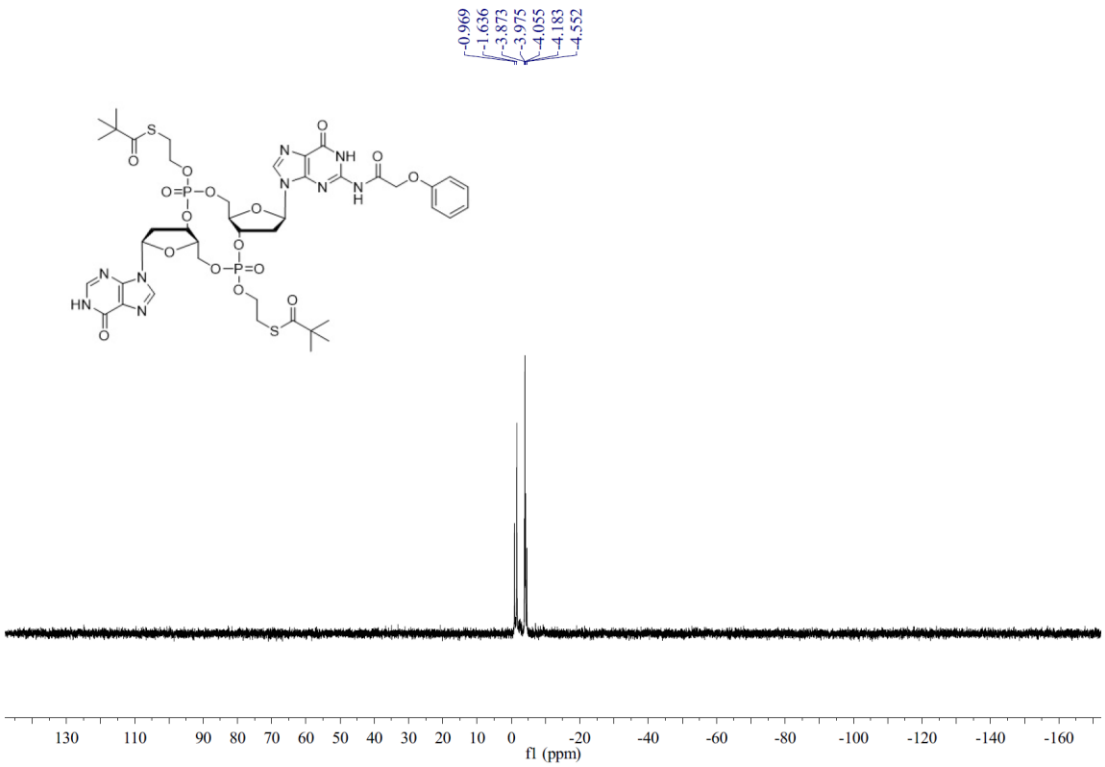

205.830
205.756
205.624
166.774
163.451
158.371
156.932
152.599
151.528
151.450
148.487
148.423
145.492
145.403
144.869
142.864
142.628
139.370
139.018
125.954
125.764
123.618
122.589
114.965
85.430
84.725
83.007
82.568
78.870
68.043
67.032
66.568
65.945
65.459
46.596
37.293
36.667
29.715
29.340
29.253
28.448
28.250
27.323
230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)


1.147
1.342
4.238
130 110 90 80 70 60 50 40 30 20 10 0 -10 -30 -50 -70 -90 -110 -130 -150
f1 (ppm)

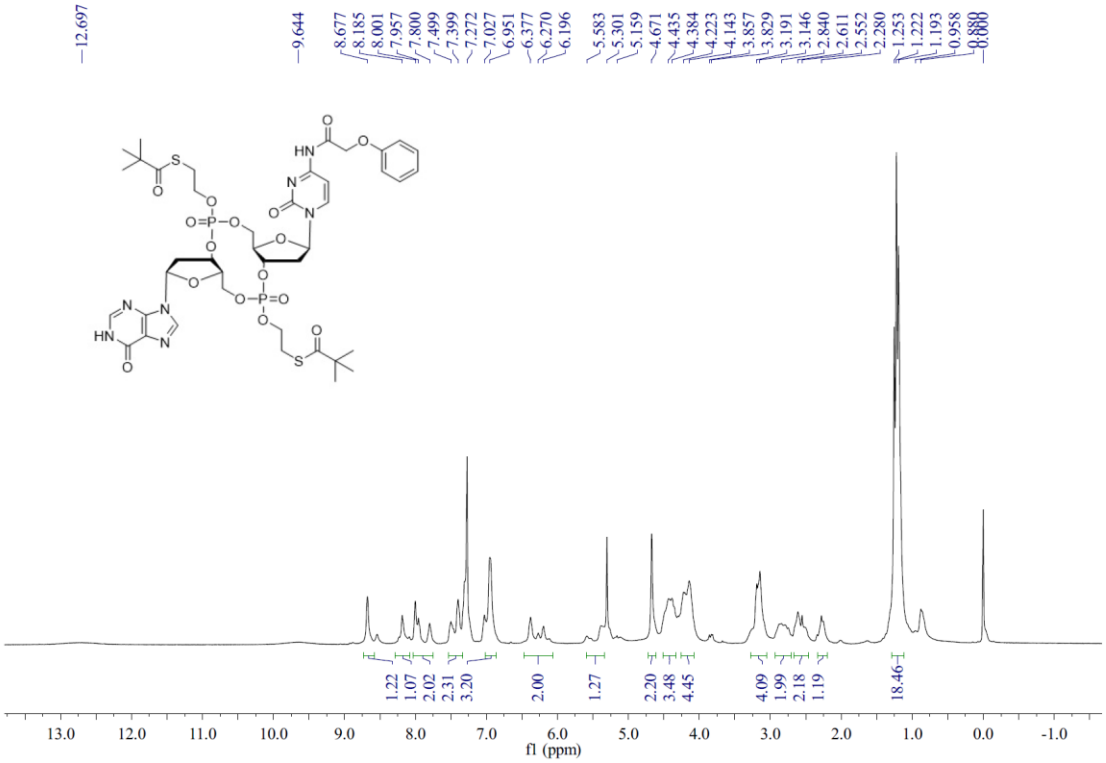
^{1}H, ^{13}C, ^{31}P NMR of **13b:**

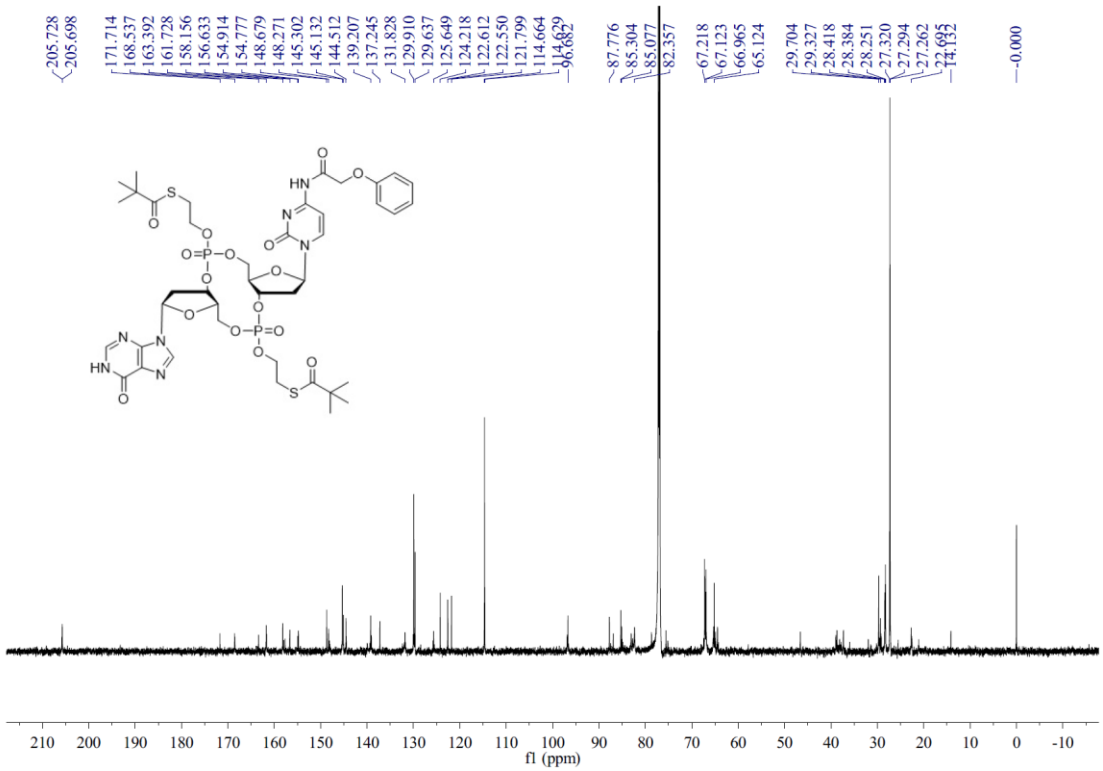


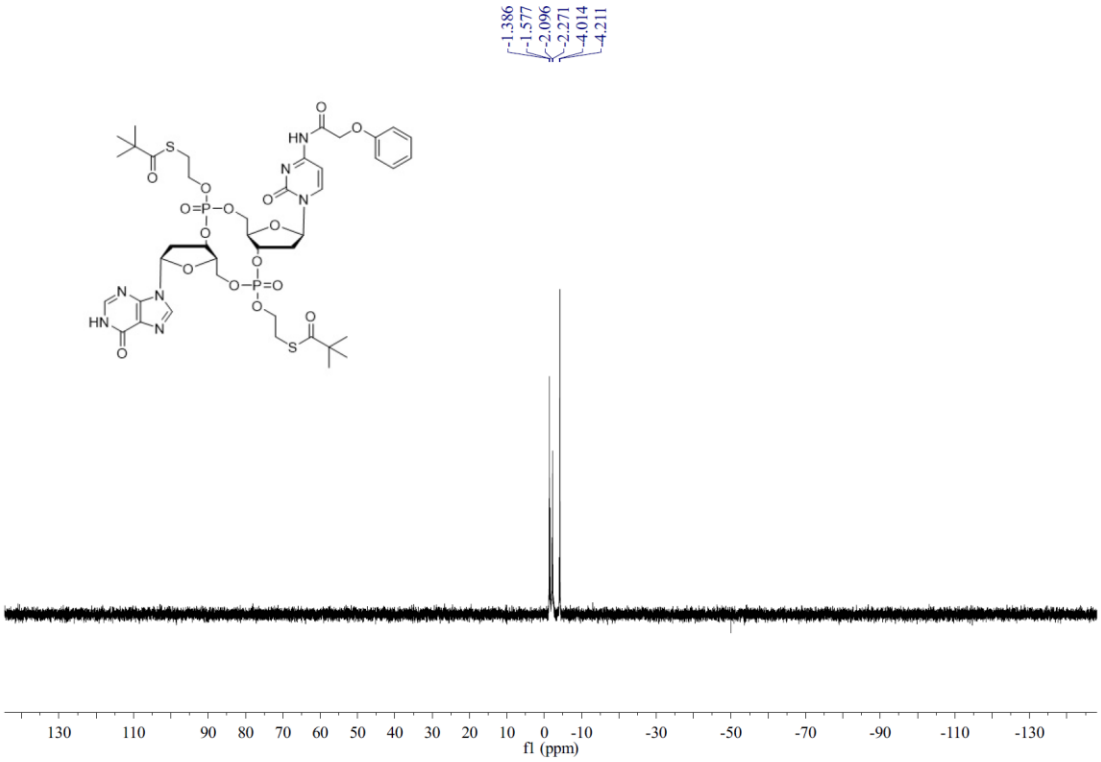




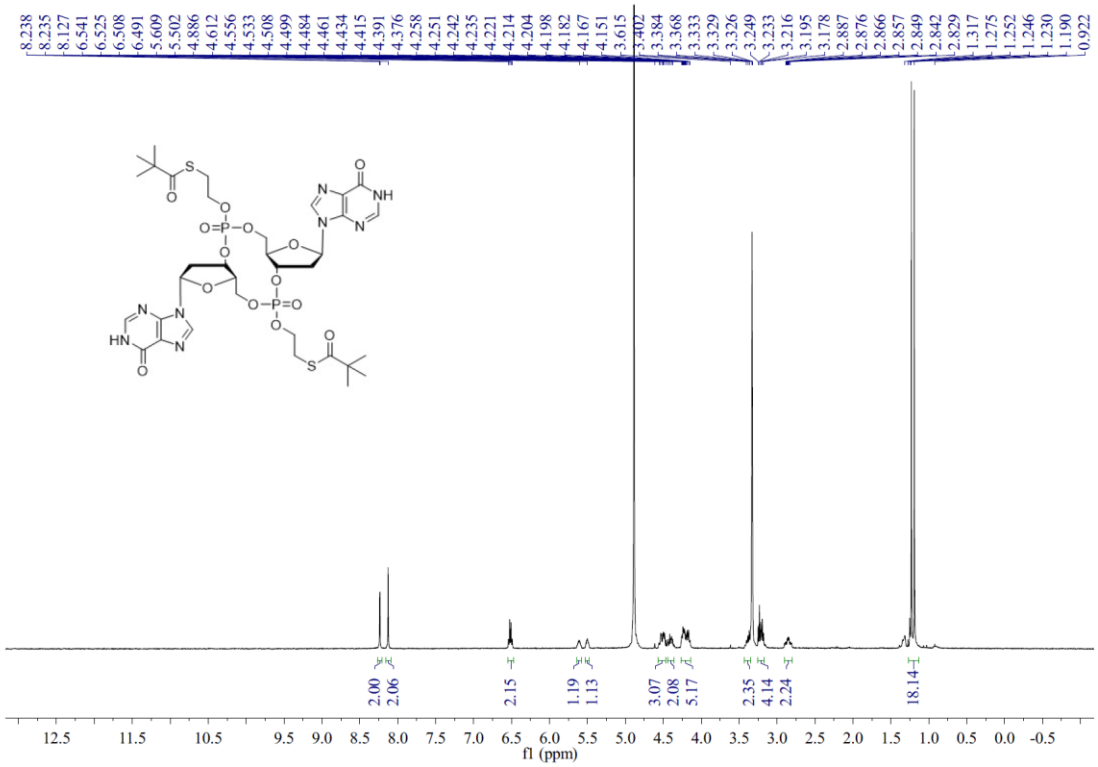
^{1}H, ^{13}C, ^{31}P NMR of **13c:**

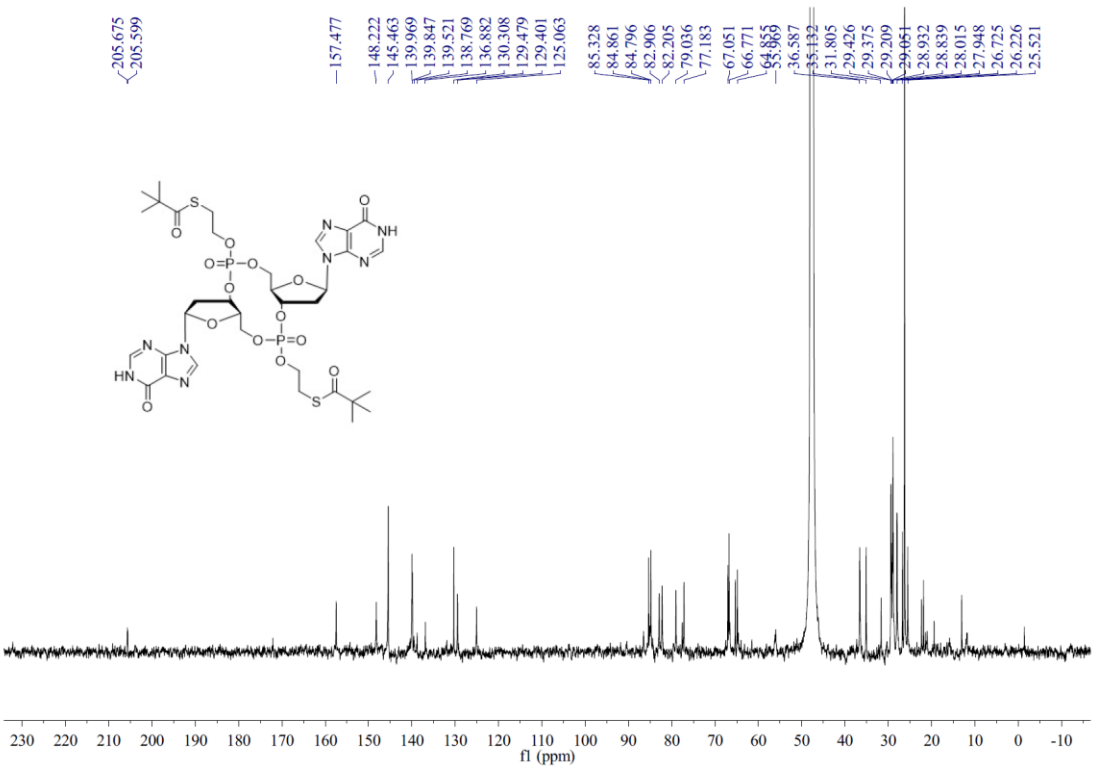


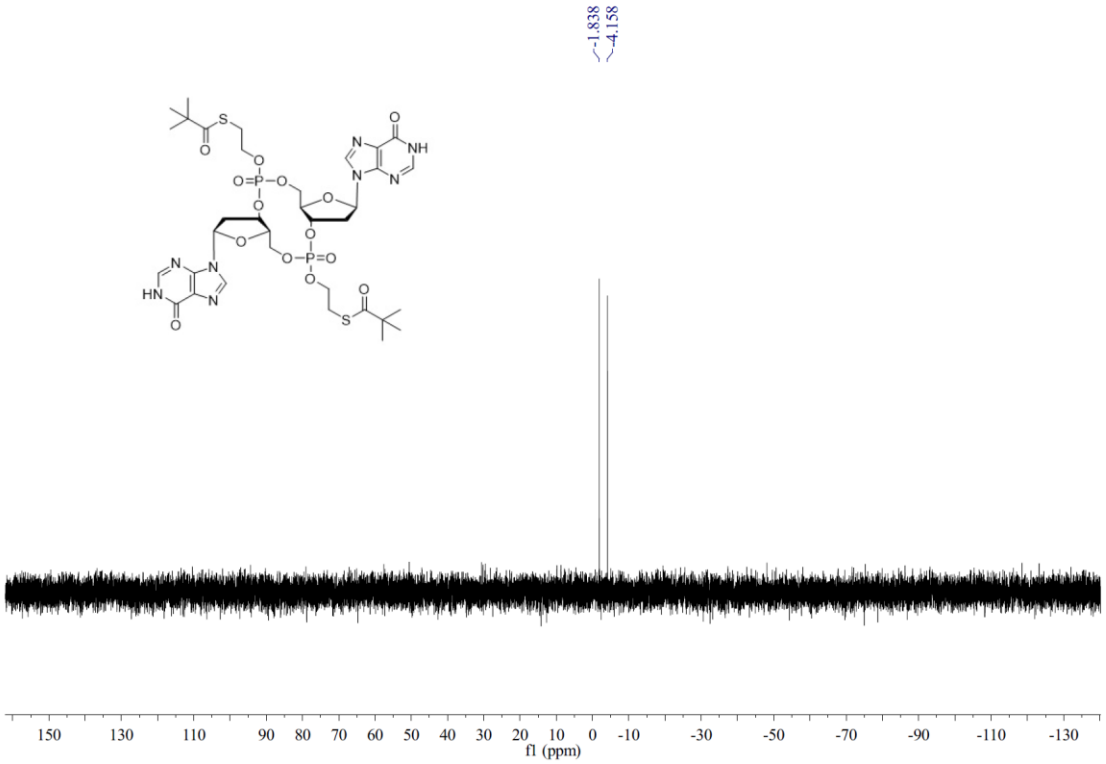

205.728
205.698
171.714
168.537
163.392
161.728
158.156
156.633
154.914
154.777
148.679
148.271
145.302
145.132
144.512
139.207
137.245
131.828
129.910
129.637
125.649
124.218
122.612
122.550
121.799
114.664
114.629
96.682
87.776
85.304
85.077
82.357
67.218
67.123
66.965
65.124
29.704
29.327
28.418
28.384
28.251
27.320
27.294
27.262
22.695
14.132
-0.000
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

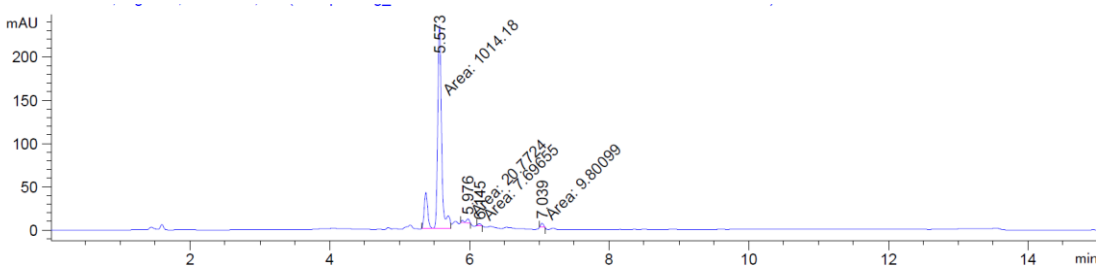

1.386
1.577
2.096
2.271
4.014
4.211
130 110 90 80 70 60 50 40 30 20 10 0 -10 -30 -50 -70 -90 -110 -130
f1 (ppm)

^{1}H, ^{13}C, ^{31}P NMR of **13d:**





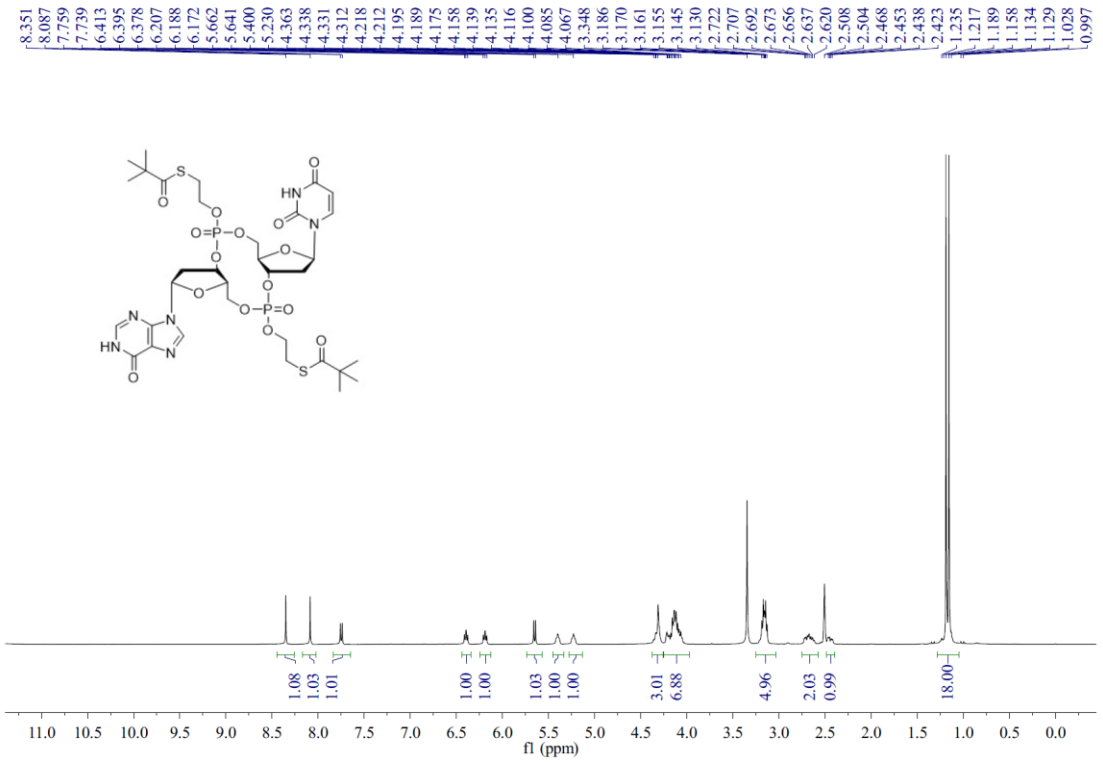


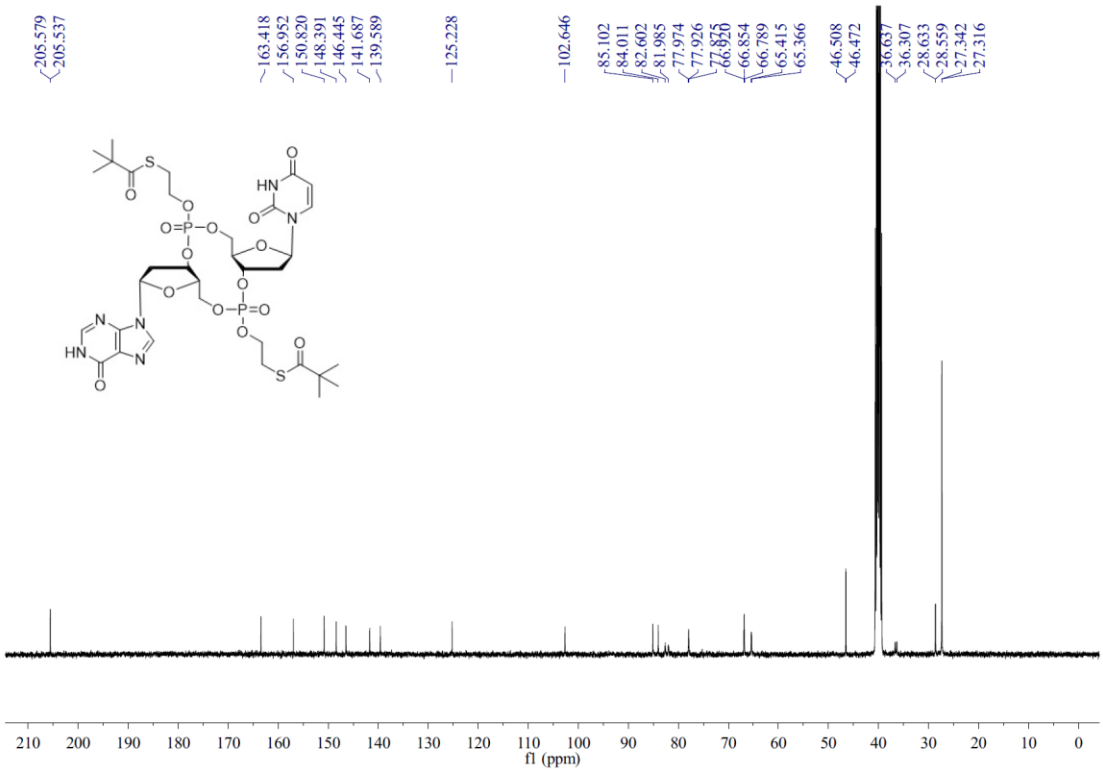


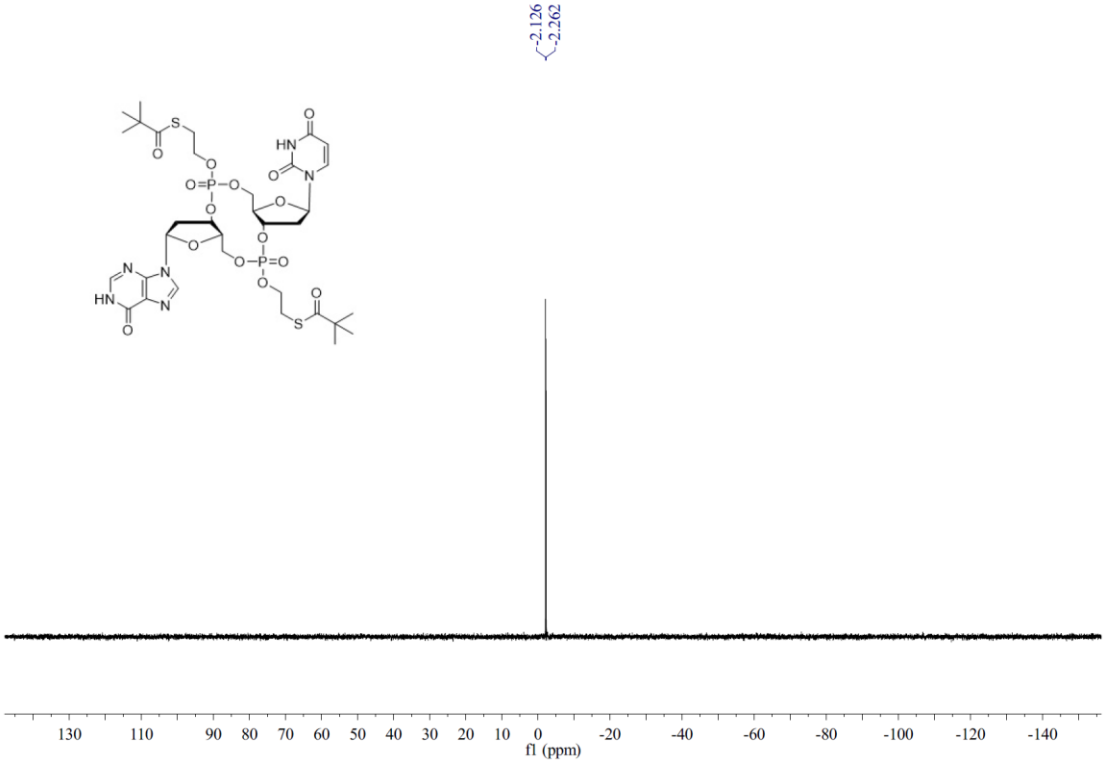
Wavelength = 254 nm

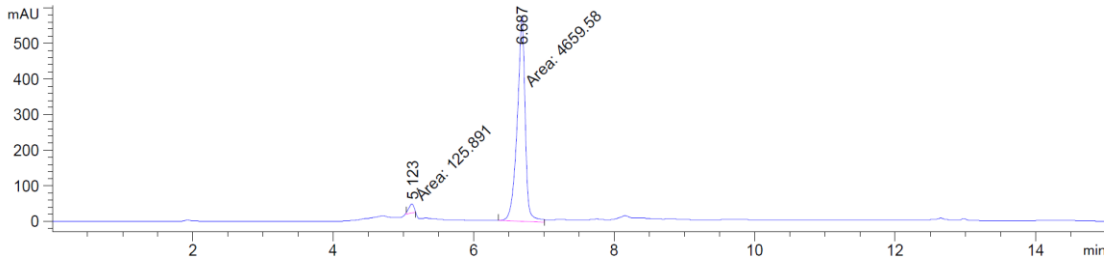
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.573	MM	0.0717	1014.18024	235.73274	96.3637
2	5.976	MM	0.0750	20.77244	4.61885	1.9737
3	6.145	MM	0.0537	7.69655	2.38733	0.7313
4	7.039	MM	0.0414	9.80099	3.94245	0.9313

^{1}H, ^{13}C, ^{31}P NMR of **13e:**





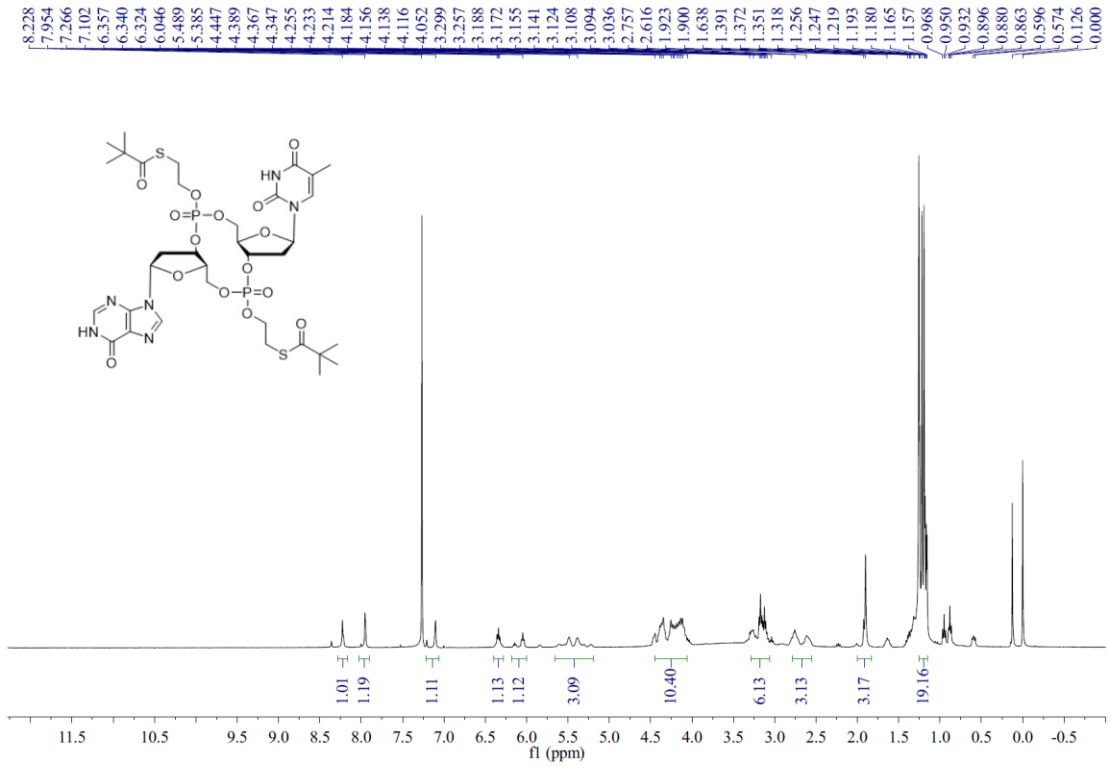


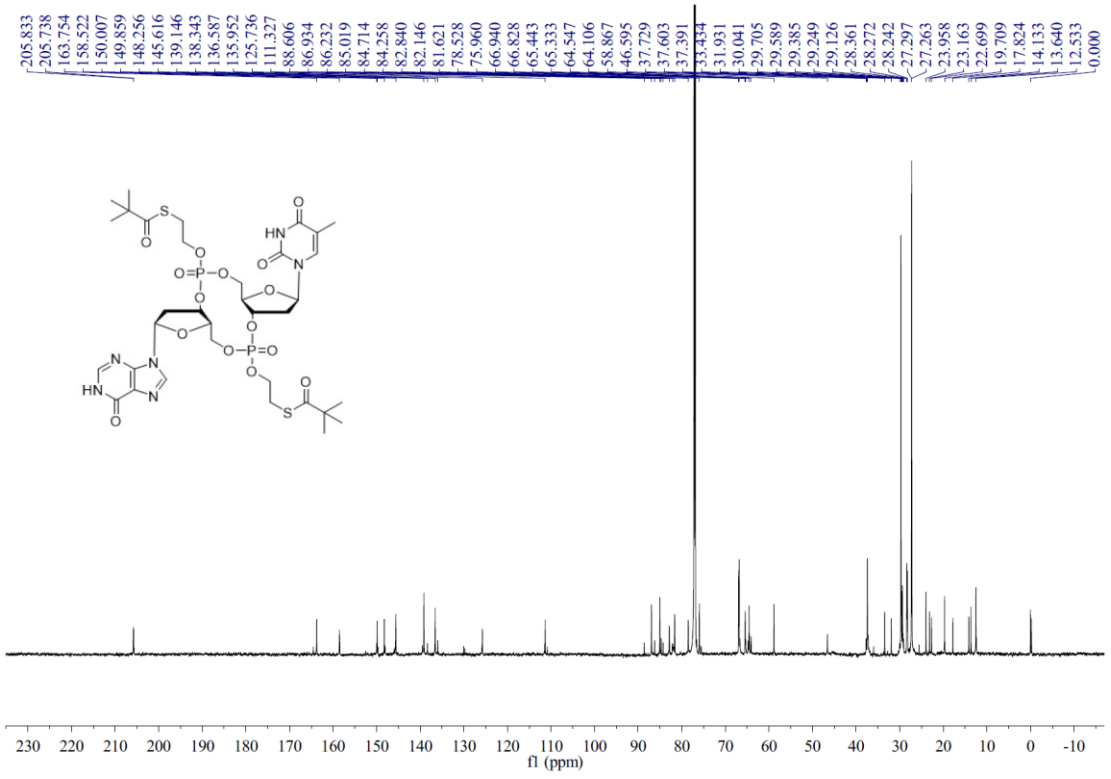


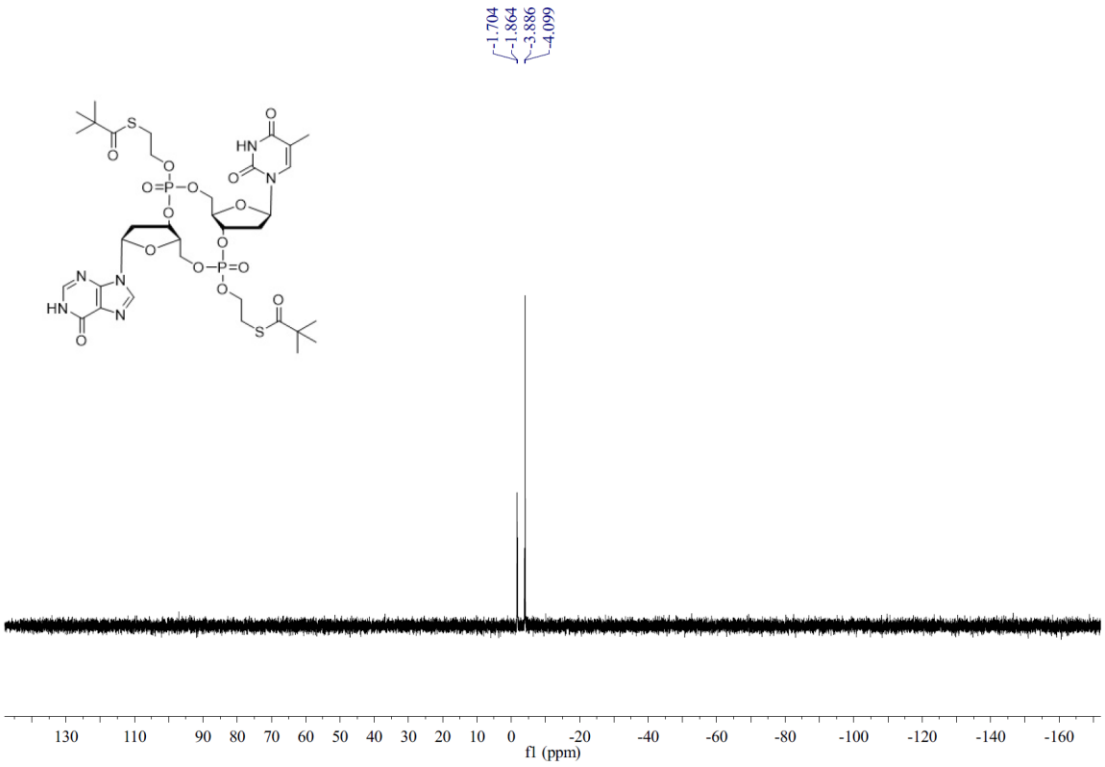
Wavelength = 254 nm

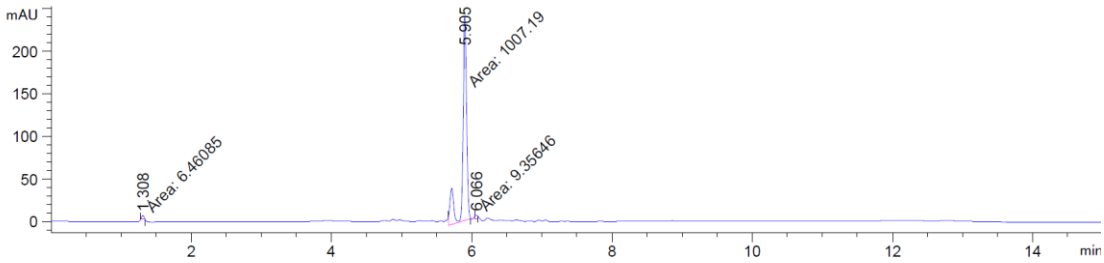
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.123	MM	0.0835	125.89102	25.13762	2.6307
2	6.687	MM	0.1344	4659.58008	577.70349	97.3693

^{1}H, ^{13}C, ^{31}P NMR of **13f:**





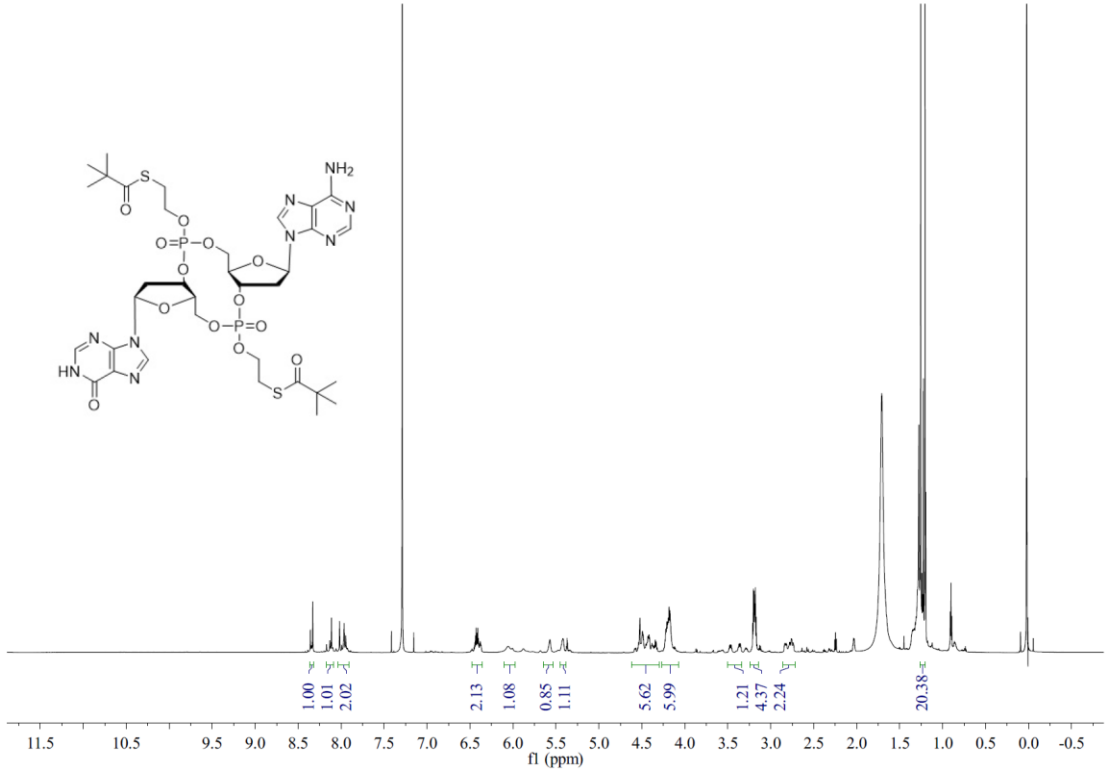


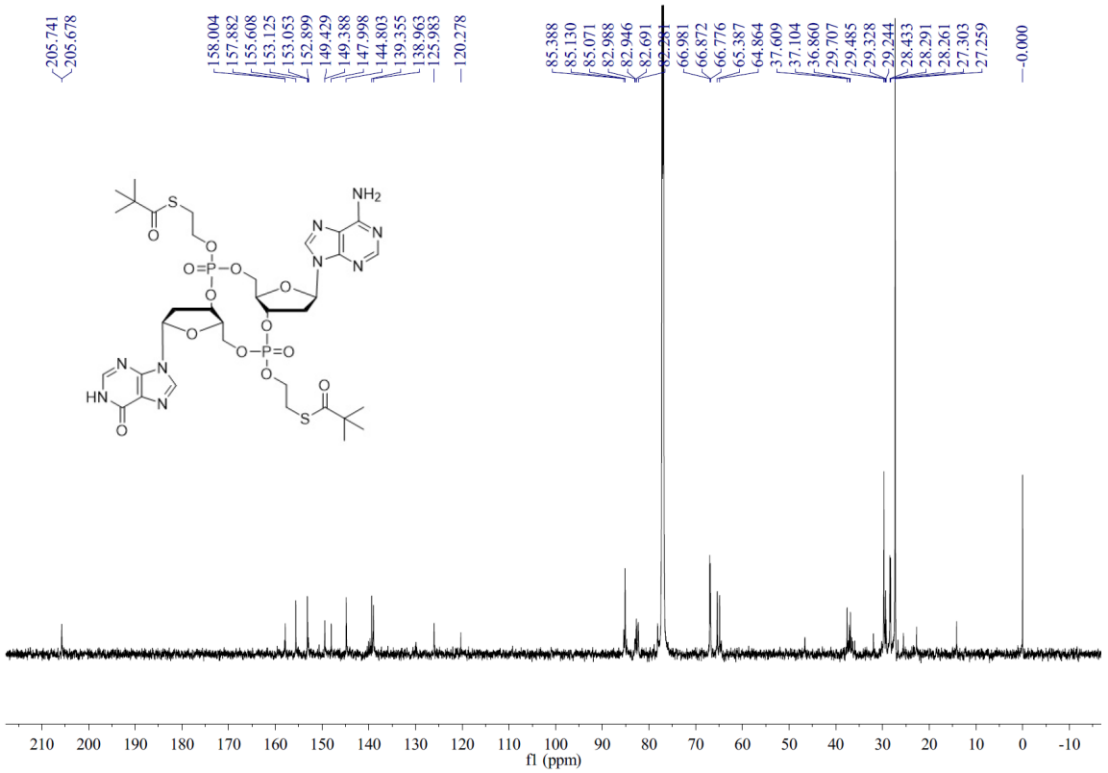


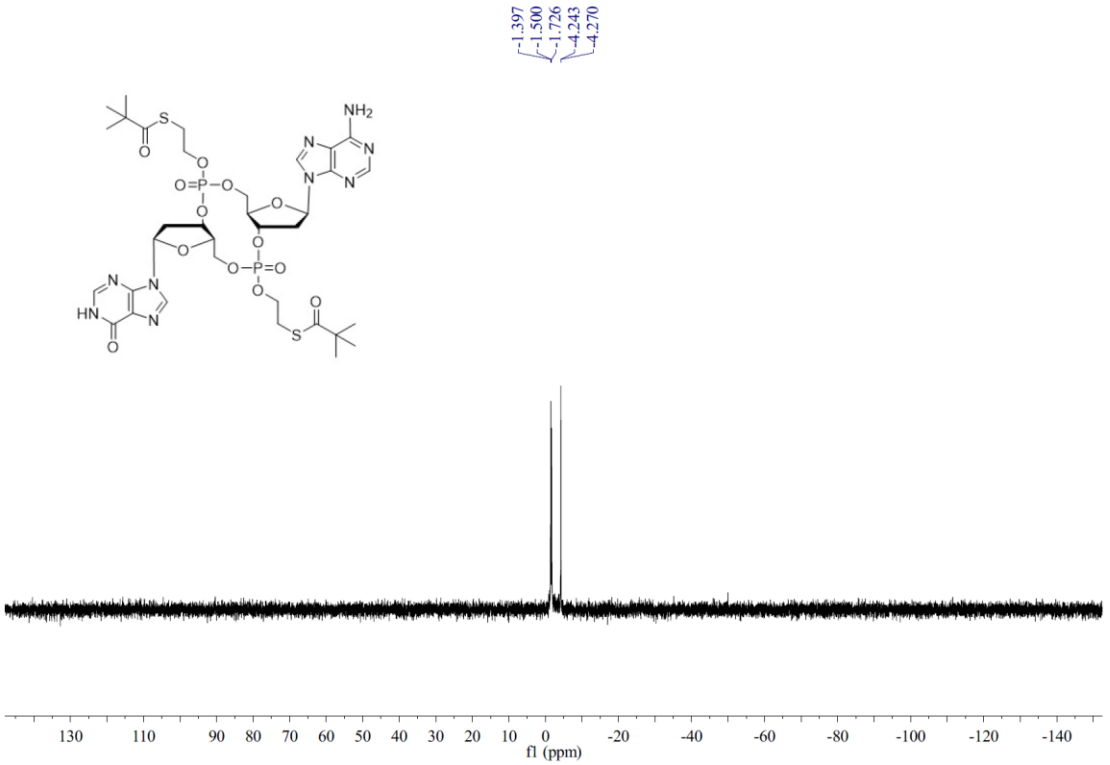
Wavelength = 254 nm

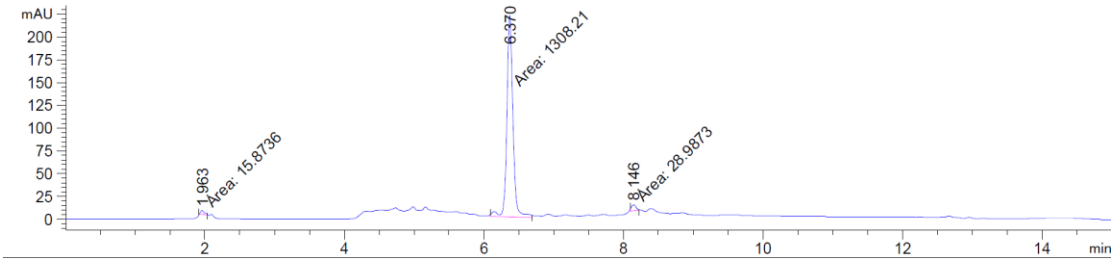
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	1.308	MM	0.0330	6.46085	3.25869	0.6316
2	5.905	MM	0.0695	1007.19232	241.39569	98.4538
3	6.066	MM	0.0353	9.35646	4.42184	0.9146

^{1}H, ^{13}C, ^{31}P NMR and HPLC spectra of **14a:**





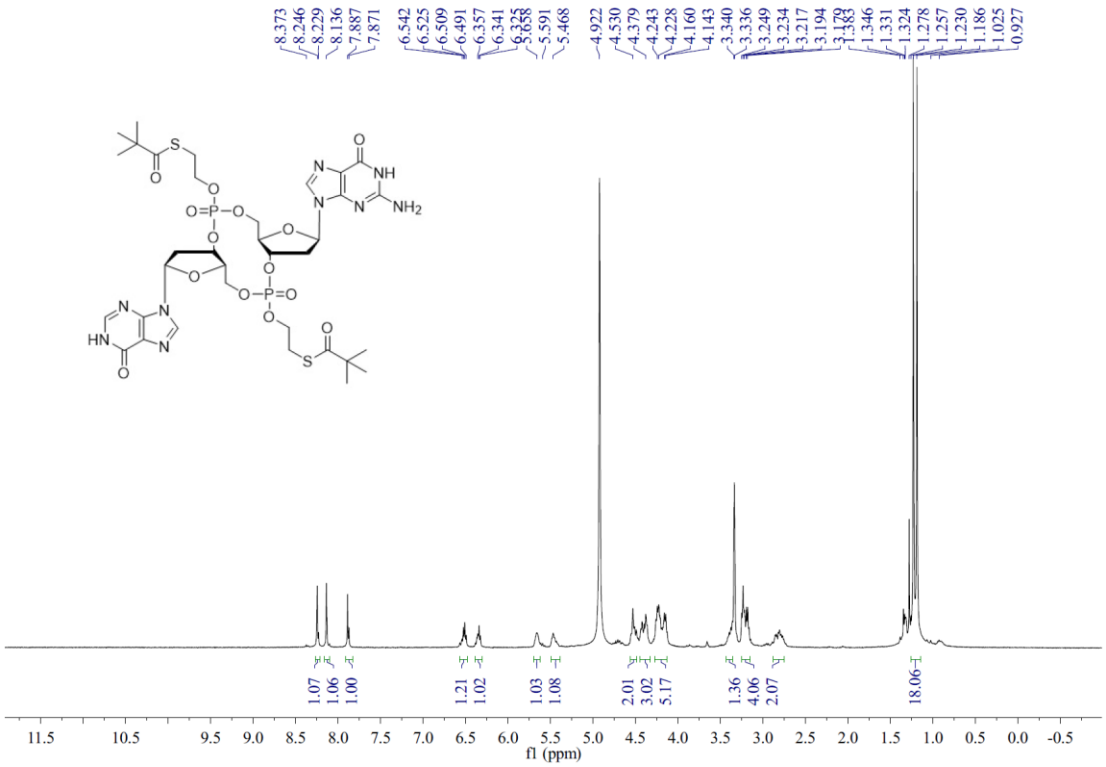


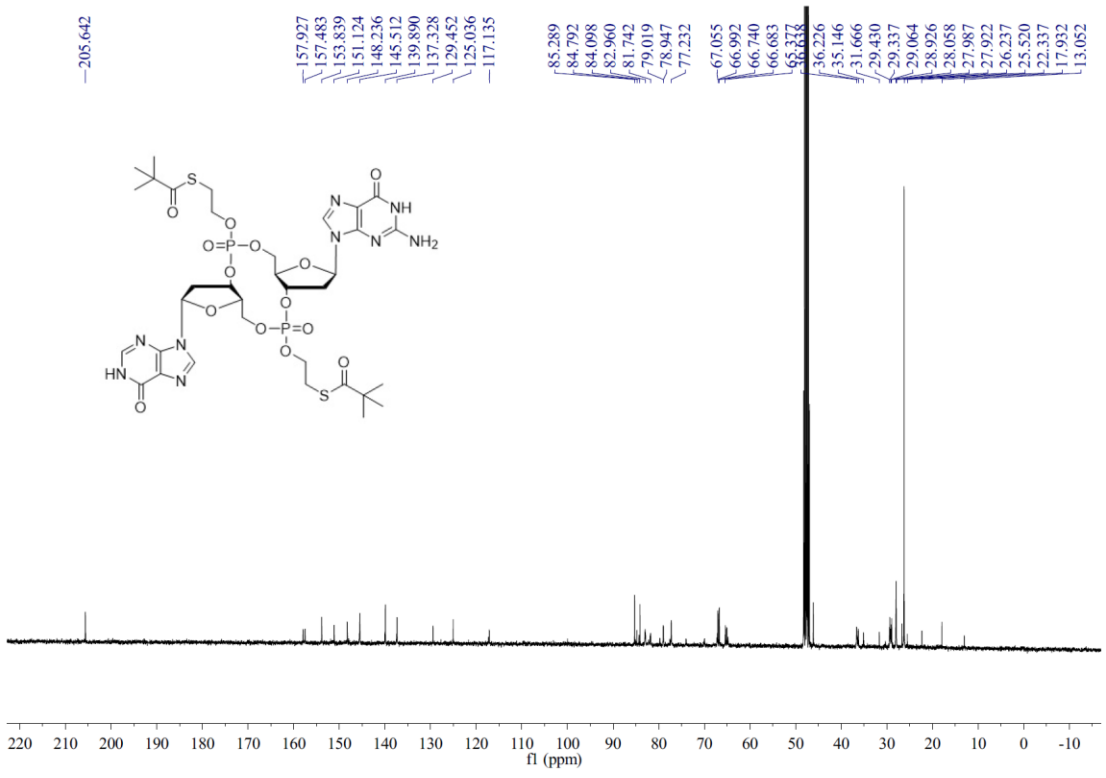


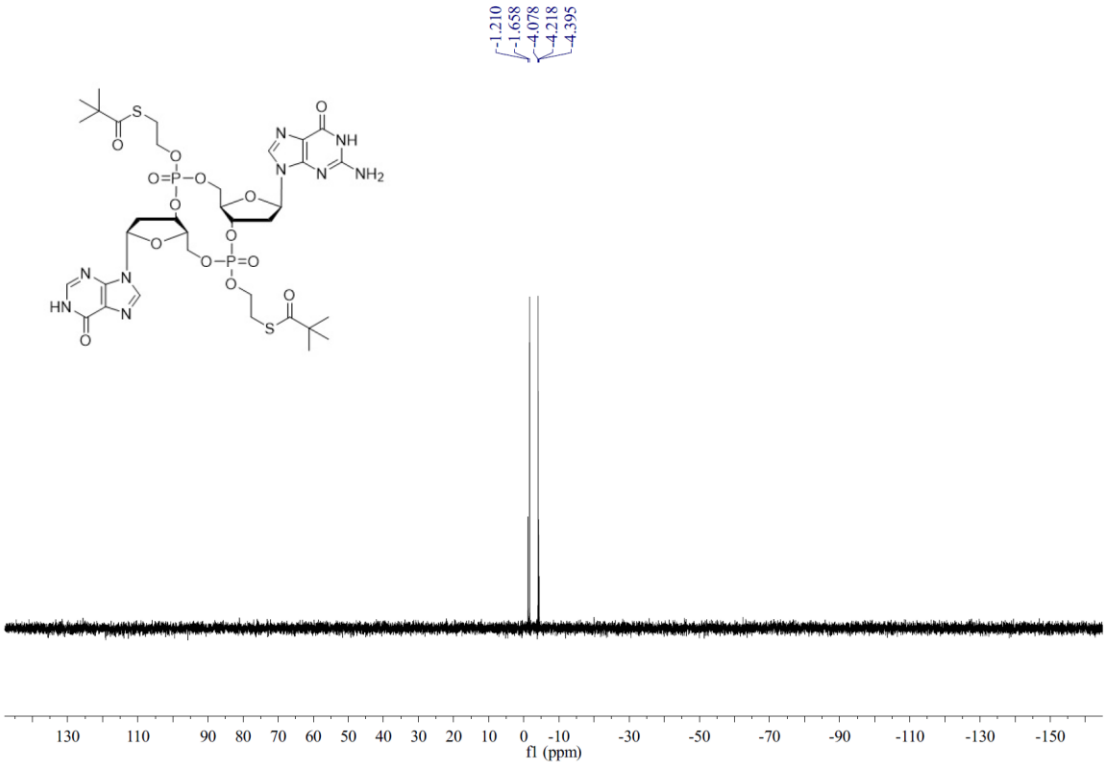
Wavelength = 254 nm

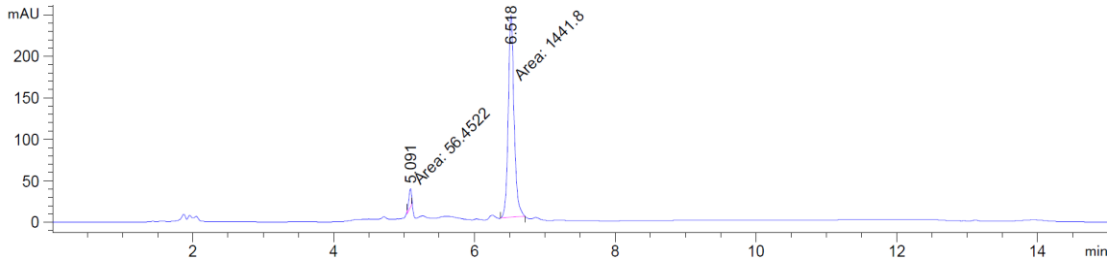
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	1.963	MM	0.0630	15.87361	4.19902	1.1732
2	6.370	MM	0.0983	1308.21338	221.85370	96.6845
3	8.146	MM	0.0730	28.98730	6.61780	2.1423

^{1}H, ^{13}C, ^{31}P NMR and HPLC spectra of **14b:**





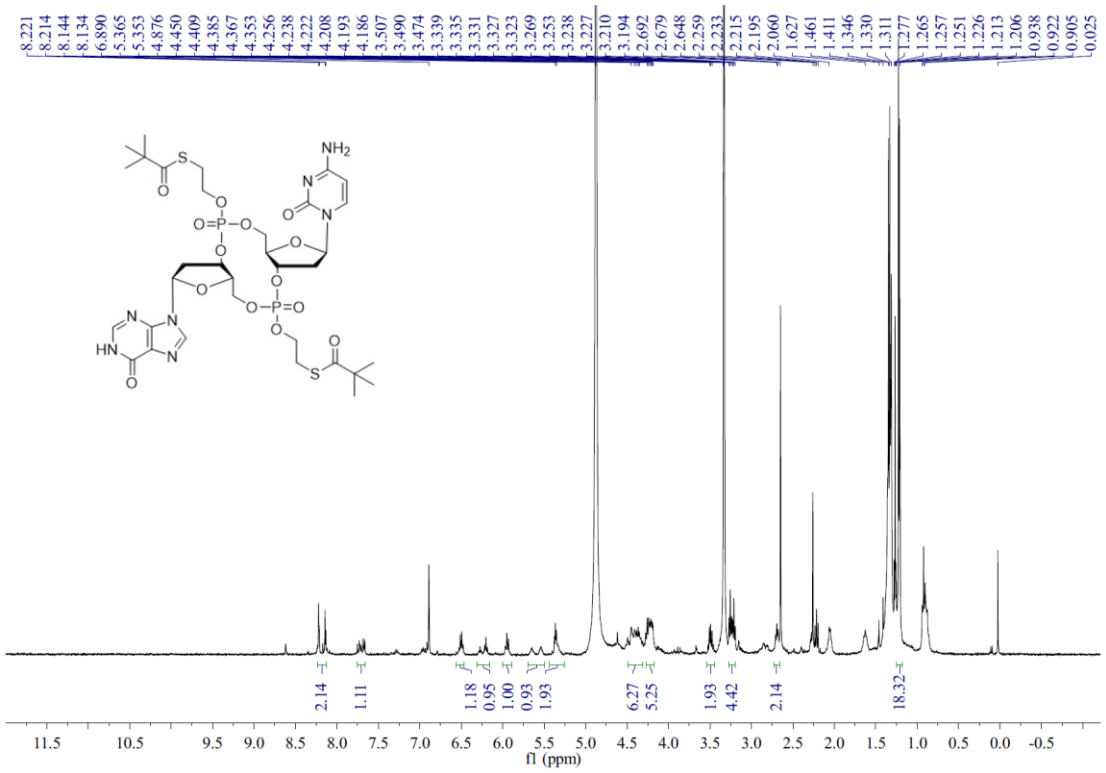


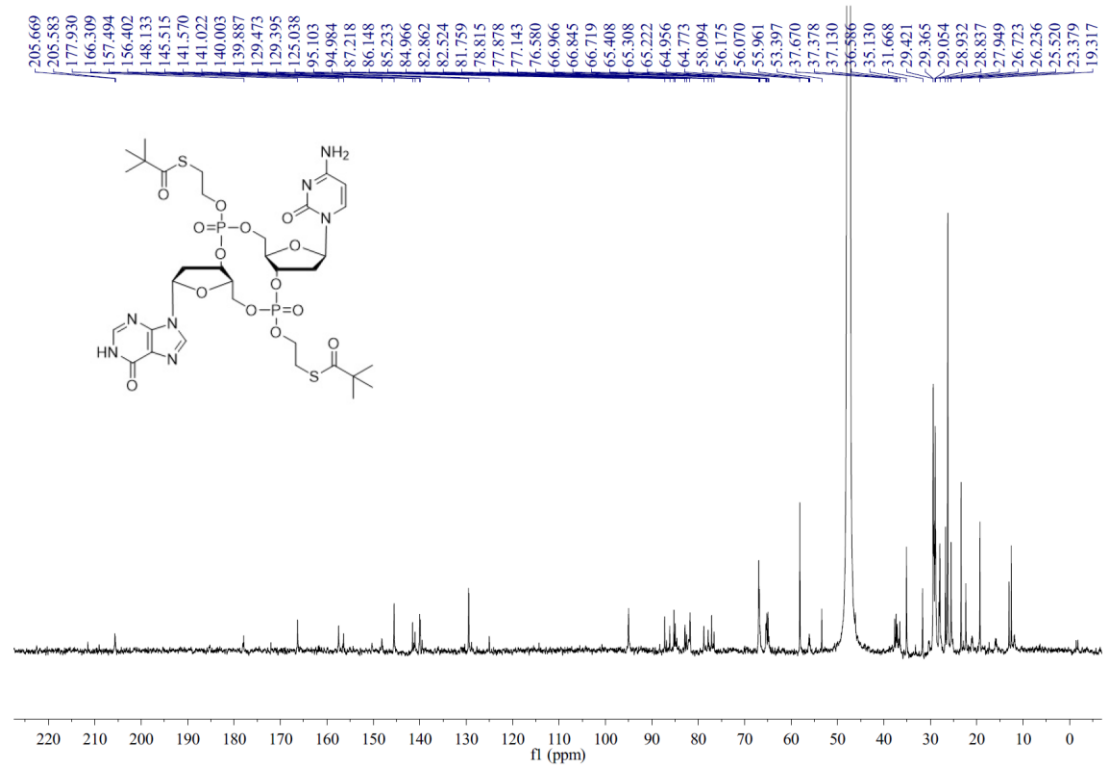


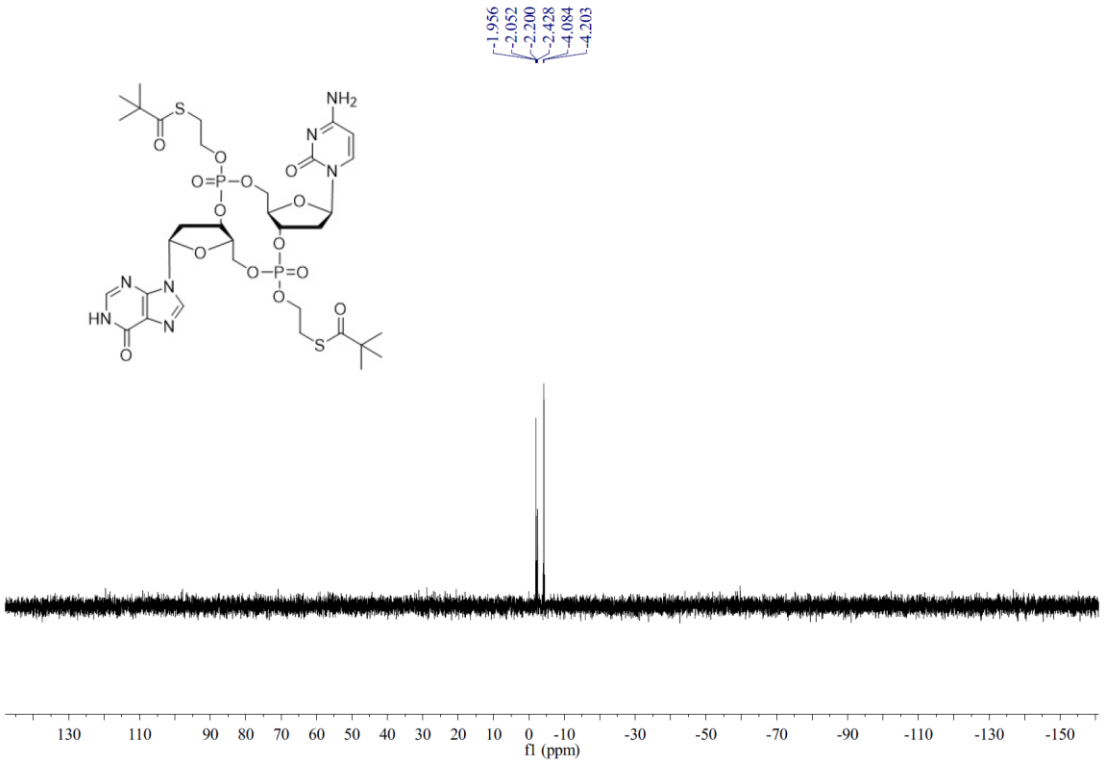
Wavelength = 254 nm

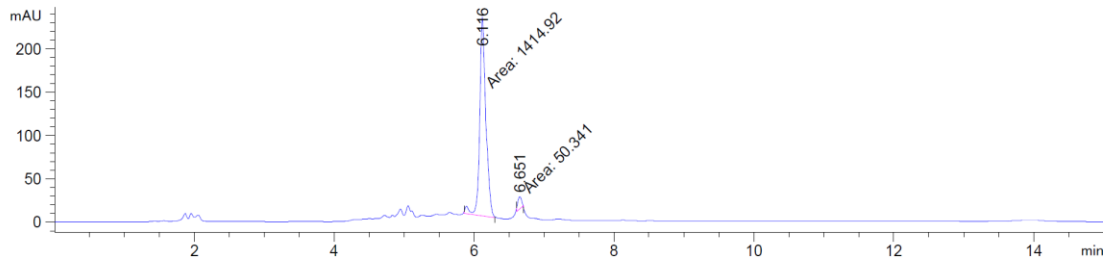
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.091	MM	0.0398	56.45222	23.61695	3.7679
2	6.518	MM	0.0985	1441.80322	244.03915	96.2321

^{1}H, ^{13}C, ^{31}P NMR and HPLC spectra of **14c:**





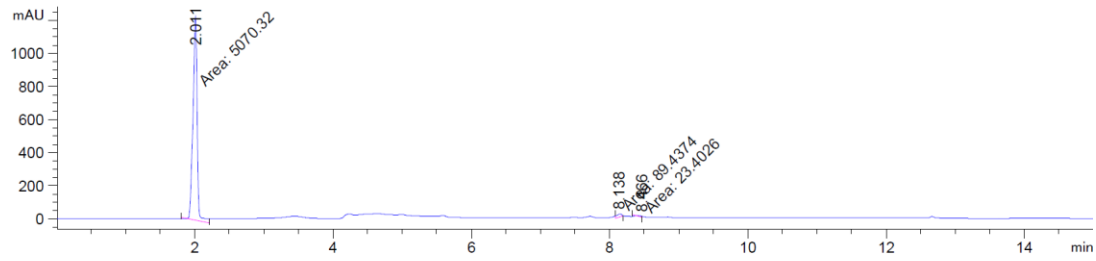




Wavelength = 254 nm

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	6.116	MM	0.1029	1414.92310	229.15054	96.5644
2	6.651	MM	0.0590	50.34100	14.21182	3.4356

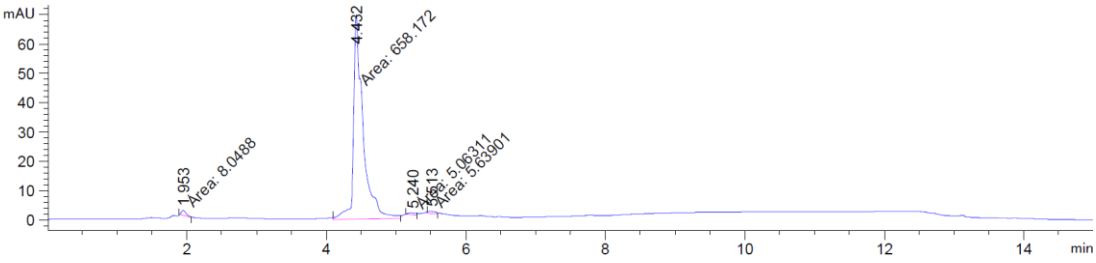
HPLC spectra of **2′,3′-cGAMP:**



Wavelength = 254 nm

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.011	MM	0.0682	5070.32373	1238.31140	97.8230
2	8.138	MM T	0.0821	89.43742	18.16607	1.7255
3	8.466	MM	0.0636	23.40262	6.13035	0.4515

HPLC spectra of **ADU-S100:**

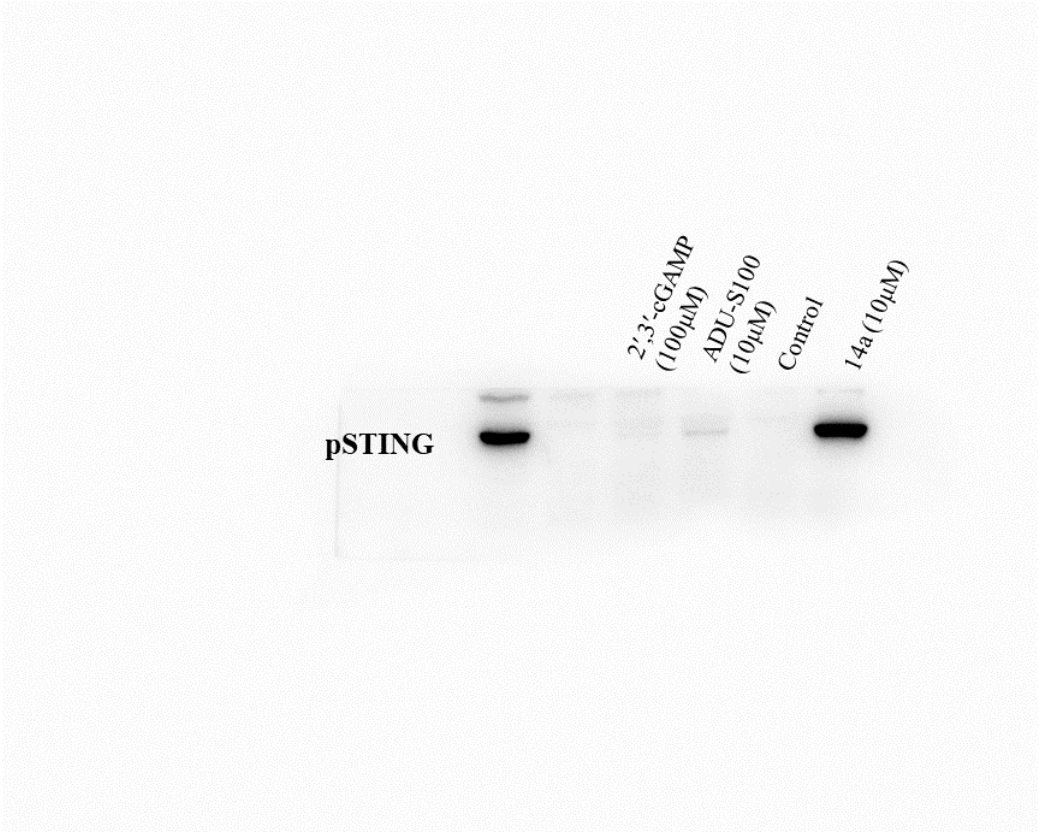


Wavelength = 254 nm

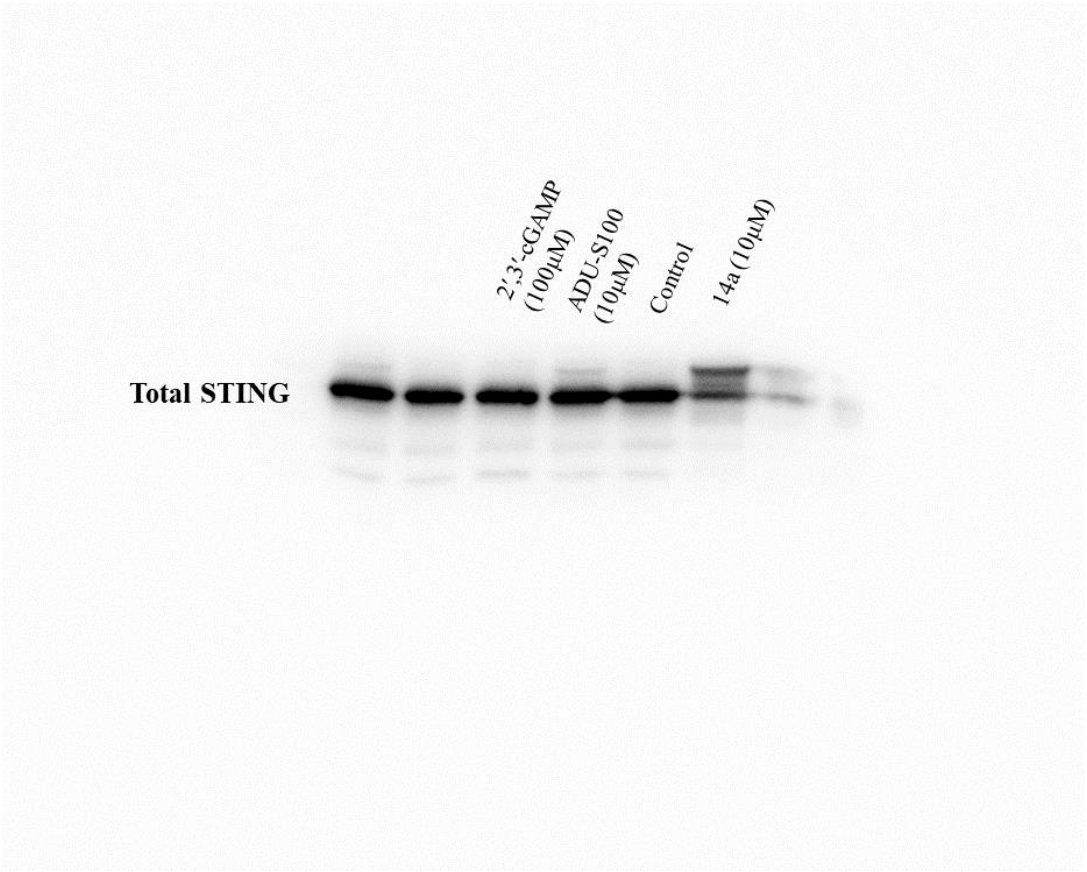
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	1.953	MM	0.0804	8.04880	1.66835	1.1890
2	4.432	MM	0.1575	658.17188	69.62640	97.2300
3	5.240	MM	0.1249	5.06311	6.75574e-1	0.7480
4	5.513	MM	0.1258	5.63901	7.46921e-1	0.8330

Raw Images of western blots:

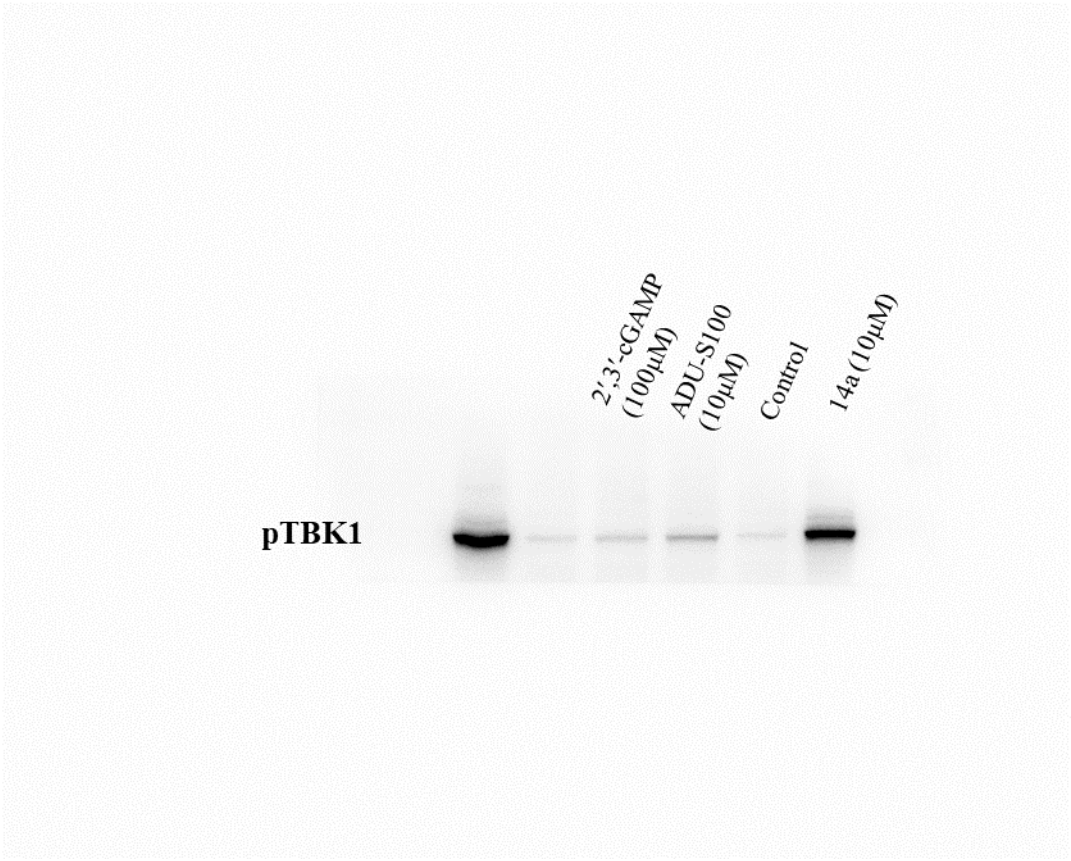
pSTING



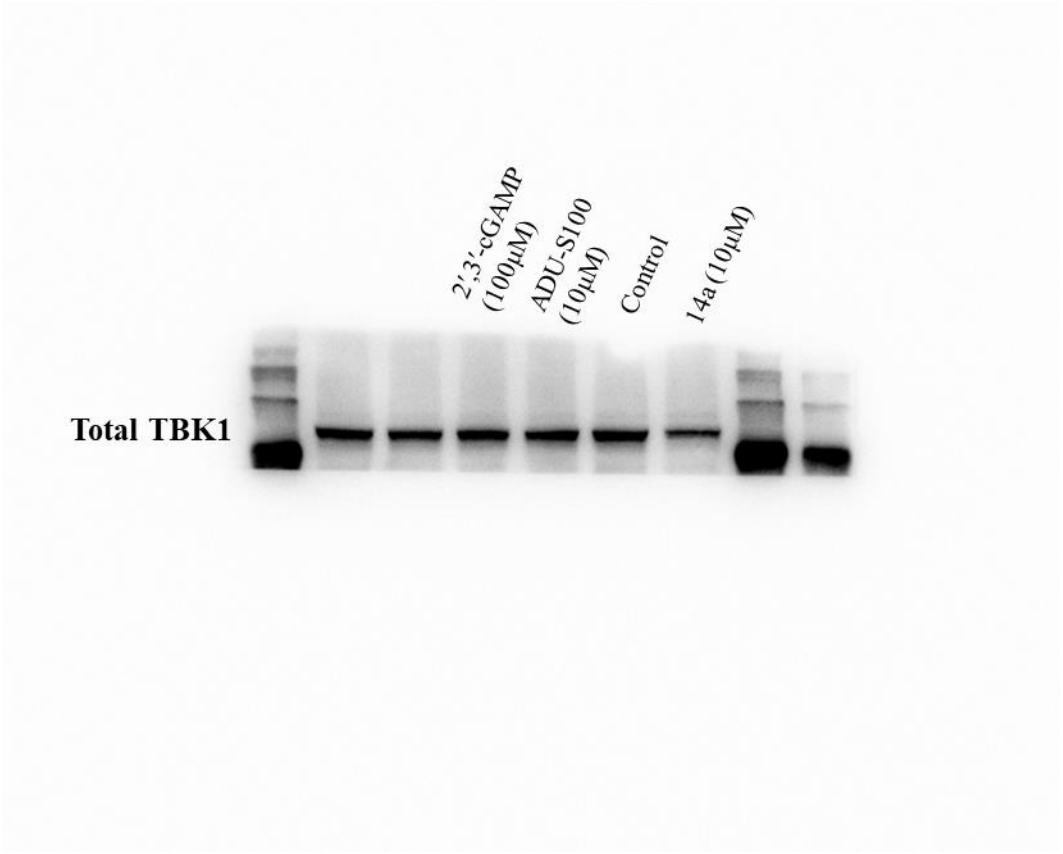
Total STING



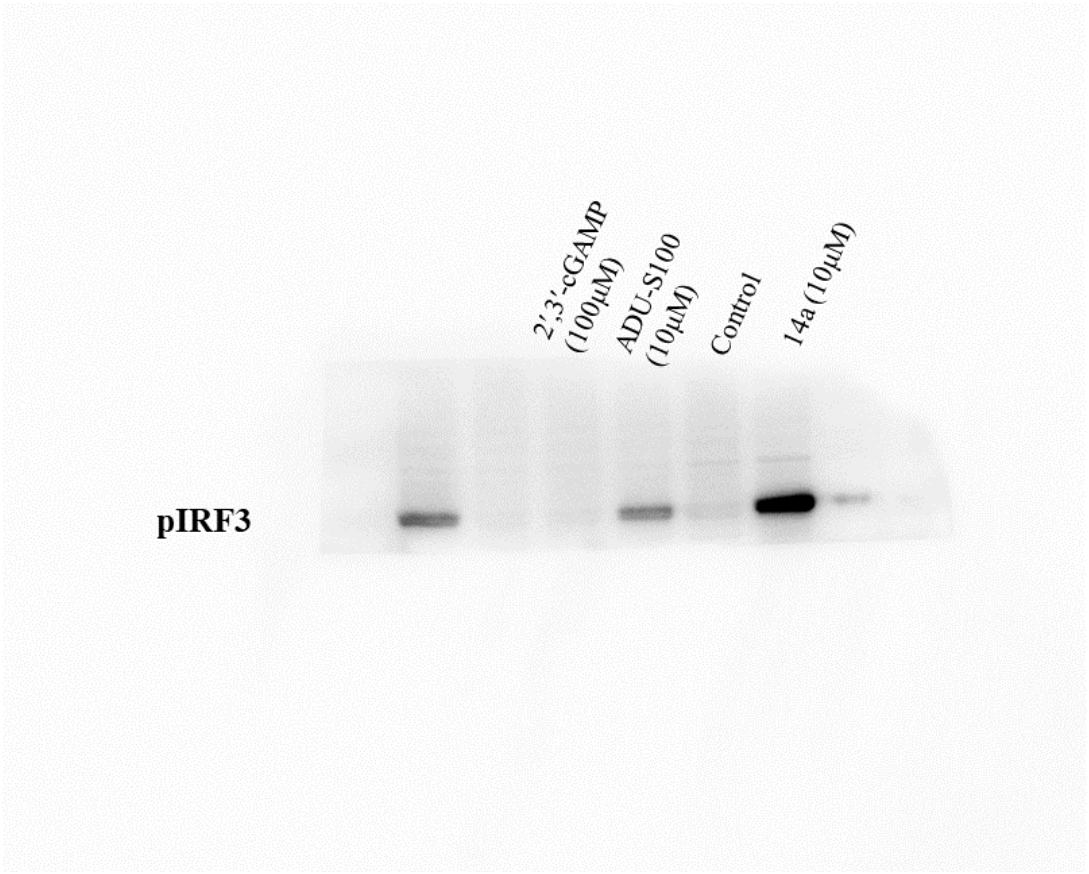
pTBK1



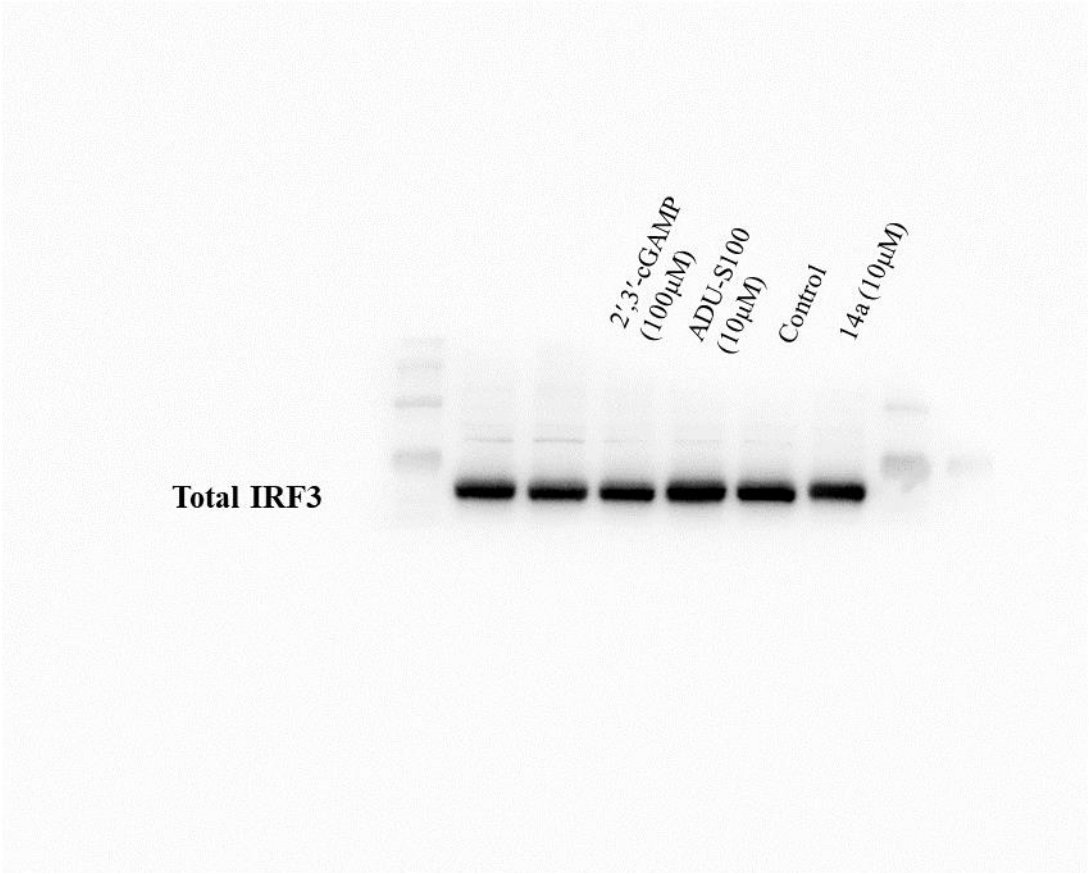
Total TBK1



pIRF3



Total IRF3



β-actin