Supporting Information

Pyrene-functionalized triazole-linked 2'-deoxyuridines - probes for discrimination of single nucleotide polymorphisms (SNPs)

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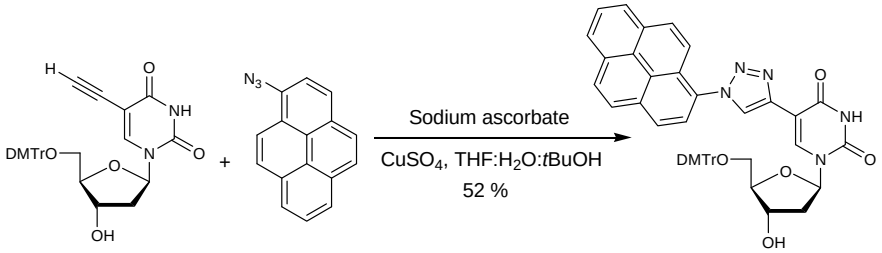
Contents

Author contributions: MEØ/PJH designed study; PK/BB synthesized starting materials; DCG/PK synthesized and characterized novel compounds; MEØ synthesized and characterized ONs; LD/AJP facilitated MS analysis of nucleosides and ONs; MEØ performed all thermal denaturation and fluorescence experiments; MEØ/PJH interpreted data and wrote main manuscript; MEØ/DCG/PK/PJH wrote supporting information; all authors reviewed manuscript/supporting information.

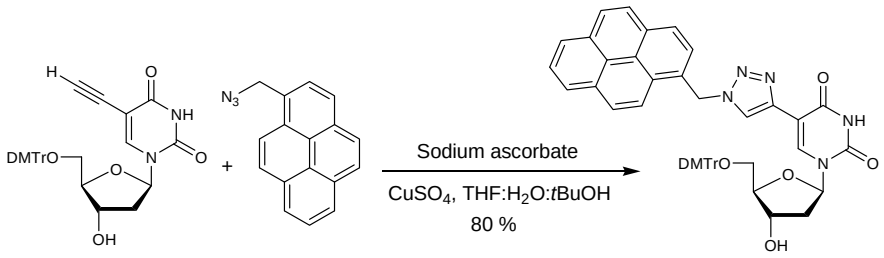
General experimental section. All reagents and solvents were of analytical grade and obtained from commercial suppliers and used without further purification. Petroleum ether of the distillation range 60-80 °C was used. Anhydrous dichloromethane, 1,2-dichloroethane and DIPEA were dried through storage over activated 4Å molecular sieves. Water content of the anhydrous solvents was checked by a Karl-Fischer apparatus. Reactions were conducted under an atmosphere of argon whenever anhydrous solvents were used. All reactions were monitored by thin-layer chromatography (TLC) using silica gel coated plates with fluorescence indicator (SiO_2-60, F-254) which were visualized a) under UV light or, b) by dipping in 5% conc. sulphuric acid in absolute ethanol (v/v) followed by heating. Silica gel column chromatography was performed with Silica gel 60 (particle size 0.040–0.063 mm, Merck) using moderate pressure (pressure ball). Silica gel columns were built with an initial starting eluent containing 1% (v/v) of Et_3N. Evaporation of solvents was carried out under reduced pressure with a temperature not exceeding 50 °C. After column chromatography, appropriate fractions were pooled, evaporated and dried at high vacuum for at least 12 h to give the obtained products in high purity (>95%) which was ascertained by 1D NMR techniques. Chemical shifts of ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz) and ^{31}P NMR (121.5 MHz) are reported in parts per million (ppm) relative to deuterated solvent or other internal standards (tetramethylsilane, and 80% phosphoric acid for ^{1}H and ^{31}P NMR, respectively). Exchangeable (ex) protons were detected by disappearance of peaks on D_2O addition. Assignments of NMR spectra are based on 2D spectra (COSY, HSQC) and DEPT-spectra. Quaternary carbons are only assigned if HSQC and DEPT spectra indicate their presence (absence of signals). MALDI-HRMS were recorded in positive ion mode on a Quadropole Time-Of-Flight tandem Mass Spectrometer (Q-TOF Premiere) equipped with a MALDI source (Waters Micromass LTD., U.K.) using 2,5-dihydroxybenzoic acid as a matrix and PEG as an internal standard.



1 **2X**

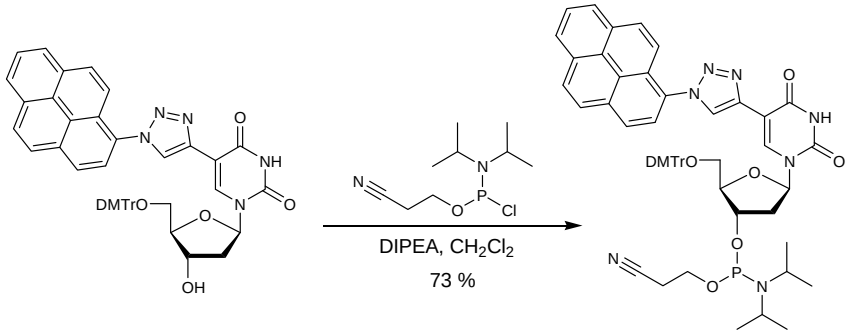
2'-Deoxy-5-(1-(pyrenyl)-1*H*-1,2,3-triazol-4-yl)uridine (2X). Nucleoside **1** (0.30 g, 0.53 mmol) was dissolved in THF:H_2O:*t*BuOH (10 mL, 3:1:1, v/v/v), and to this was added aq. sodium ascorbate (1M, 1.2 mL, 1.2 mmol), aq. $CuSO_4$ (1.1 mL, 7.5%, w/v, 0.32 mmol), and 1-azidopyrene (200 mg, 0.80 mmol). The solution was stirred at rt for 4 h, whereupon it was diluted with EtOAc (30 mL) and brine (30 mL) and the phases were separated. The organic phase was washed with sat. aq. $NaHCO_3$ (30 mL), and the combined aqueous phase was back-extracted with EtOAc (30 mL). The combined organic phase was dried (Na_2SO_4), evaporated at reduced pressure, and the resulting crude purified by column chromatography (0-100% EtOAc in petroleum ether, v/v) to afford **2X** (220 mg, 52%) as a yellow solid. R_f = 0.3 (70% EtOAc in petroleum ether, v/v); MALDI-HRMS *m/z* 820.2701 ($[M+Na]^+$, $C_{48}H_{39}N_5O_7Na^+$ calc. 820.2742). ^{1}H NMR ($CDCl_3$) δ 9.24 (s, 1H, ex, NH) 8.69 (s, 1H, H-triazole), 8.66 (s, 1H, H6), 8.27 (d, 1H, *J* = 7.5 Hz, Ar), 8.22-8.25 (m, 2H, Ar), 8.16-8.18 (d, 1H, *J* = 9.0 Hz, Ar), 8.09-8.10 (m, 2H, Ar), 8.06-8.07 (d, 1H, *J* = 7.5 Hz, Ar), 8.04 (d, 1H, *J* = 8.0 Hz, Ar), 7.86 (d, 1H, *J* = 9.0 Hz, Ar) 7.46-7.48 (m, 2H, Ar), 7.37-7.39 (m, 4H, Ar), 7.27-7.30 (m, 2H, Ar) 7.17-7.20 (m, 1H, Ar) 6.85 (d, 4H, *J* = 9.0 Hz, Ar), 6.38 (t, 1H, *J* = 6.5 Hz, H1'), 4.49-4.52

(m, 1H, H3'), 4.10-4.13 (m, 1H, H4'), 3.73 (s, 6H, CH_3O), 3.55-3.58 (m, 1H, H5'), 3.48-3.51 (m, 1H, H5'), 2.56-2.59 (m, 1H, H2'$_a$), 2.49 (bs, 1H, ex, 3'-OH), 2.37-2.42 (m, 1H, H2'$_b$); ^{13}C NMR ($CDCl_3$, 75.5 mHz) δ 161.0 (C), 158.6 (C), 149.5 (C), 144.6 (C), 139.0 (C), 136.4 (C6), 135.7 (C), 135.6 (C), 132.2 (C), 131.1 (C), 130.7 (C), 130.4 (C), 130.1 (Ar), 130.0 (Ar), 129.6 (Ar), 129.1 (Ar), 128.9 (Ar), 128.1 (Ar), 127.9 (Ar), 126.9 (Ar), 126.8 (Ar), 126.7 (Ar), 126.3 (Ar), 126.1 (C), 126.0 (Ar), 125.1 (C), 125.0 (CH-triazole), 124.7 (Ar), 124.2 (C), 123.3 (Ar), 121.3 (Ar), 113.3 (Ar), 106.1 (C), 86.9 (C), 85.91 (C4'), 85.88 (C1'), 72.6 (C3'), 63.8 (C5'), 55.2 (OCH_3), 40.5 (C2').



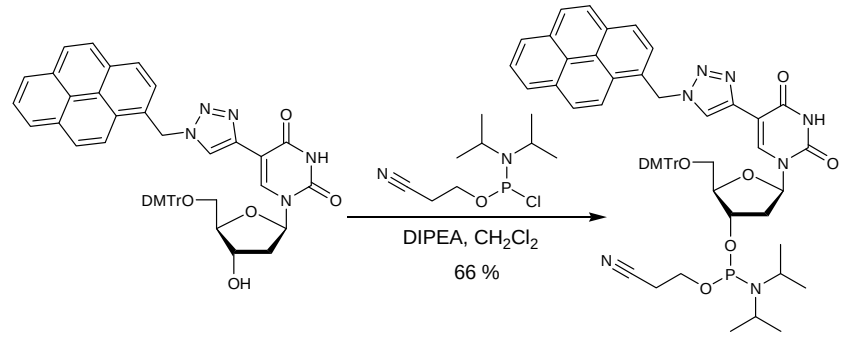
1 **2Y**

2'-Deoxy-5-(1-(pyrenmethyl)-1*H*-1,2,3-triazol-4-yl)uridine (2Y). To a solution of nucleoside **1** (86 mg, 0.15 mmol) and 1-azidomethylpyrene (58 mg, 0.21 mmol) in THF:H_2O:*t*BuOH (5 mL, 3:1:1, v/v/v) was added aq. sodium ascorbate (1M, 280 μL, 0.28 mmol), and aq. $CuSO_4$ (260 μL, 7.5%, w/v, 0.077 mmol). The solution was stirred at rt for 4 h, whereupon it was diluted with EtOAc (20 mL) and brine (20 mL) and the phases were separated. The organic phase was washed with sat. aq. $NaHCO_3$ (20 mL), and the combined aqueous phase was back-extracted with EtOAc (20 mL). The combined organic phase was dried (Na_2SO_4), evaporated at reduced pressure, and the resulting crude purified by column chromatography (0-100% EtOAc in petroleum ether, v/v) to afford **2X** (99 mg, 80%) as a slightly yellow solid material. R_f = 0.7 (80% EtOAc in petroleum ether, v/v); MALDI-HRMS *m/z* 818.3181 ($[M+Li]^+$, $C_{49}H_{41}N_5O_7Li^+$ calc. 818.3161). ^{1}H NMR (DMSO-d_6) δ 11.67 (s, 1H, ex, NH), 8.56 (d, 1H, *J* = 9.0 Hz, Ar), 8.39 (s, 1H, H-triazole), 8.34 (d, 2H, *J* = 7.5 Hz, Ar), 8.31 (d, 1H, *J* = 8.0, Ar), 8.29 (s, 1H, H6), 8.27 (d, 1H, *J* = 9.0 Hz, Ar), 8.22 (d, 1H, *J* = 9.0 Hz, Ar), 8.19 (d, 1H, *J* = 9.0 Hz, Ar), 8.11 (t, 1H, *J* = 7.5 Hz, Ar), 8.05 (d, 1H, *J* = 7.5 Hz, Ar) 7.32-7.33 (m, 2H, Ar), 7.21-7.23 (m, 4H, Ar), 7.15-7.18 (m, 2H, Ar), 7.01-7.04 (m, 1H, Ar), 6.81 (d, 4H, *J* = 8.5 Hz, Ar), 6.42 (s, 2H, CH_2), 6.14 (t, 1H, *J* = 6.5 Hz, H1'), 5.31 (d, 1H, ex, *J* = 4.5 Hz, 3'-OH), 4.13- 4.15 (m, 1H, H3'), 3.91-3.93 (m, 1H, H4'), 3.62 (s, 3H, OCH_3), 3.61 (s, 3H, OCH_3) 3.18-3.19 (m, 2H, H5'), 2.17-2.27 (m, 2H, H2'); ^{13}C NMR (DMSO-d_6) δ 161.0 (C), 157.9 (C), 149.4 (C), 144.7 (C), 138.8 (C), 135.41 (C6), 135.36 (C), 135.34 (C), 131.0 (C), 130.7 (C), 130.1 (C), 129.60 (Ar), 129.59 (Ar), 129.1 (C), 128.4 (C), 128.2 (Ar), 127.7 (Ar), 127.6 (Ar), 127.53 (Ar), 127.50 (Ar), 127.2 (Ar), 126.43 (Ar), 126.40 (Ar) 125.6 (Ar), 125.5 (Ar), 125.0 (Ar), 123.9 (Ar), 123.7 (Ar), 122.7 (Ar), 122.3 (CH-triazole), 113.1 (Ar), 105.0 (C), 85.7(C), 85.6 (C4'), 85.1 (C1'), 70.4 (C3'), 63.6 (C5'), 54.8 (OCH_3), 50.8 (CH_2), 39.7 (C2').



2X **3X**

2'-Deoxy-5-(1-(pyrenyl)-1*H*-1,2,3-triazol-4-yl)uridine phosphoramidite (3X). Nucleoside **2X** (0.20 g, 0.24 mmol) was coevaporated in anh. 1,2-dichloroethane (3×10mL) and redissolved in anh. CH_2Cl_2 (4.0 mL). To this solution was dropwise added DIPEA (170 μL, 0.98 mmol) and 2-cyanoethyldiisopropyl-chlorophosporamidite (76 μL, 0.34 mmol) and stirred at rt for 3.5 h, whereupon the solvent was evaporated under reduced pressure, and the resulting crude was purified via column chromatography (0–3% MeOH in CH_2Cl_2, v/v) to afford amidite **3X** (0.18 g, 73%) as a slightly yellow solid. R_f = 0.3 (5% MeOH in CH_2Cl_2, v/v); MALDI-HRMS *m/z* 1020.3821 ($[M+Na]^+$, $C_{57}H_{56}N_7O_8P$ Na^+ calc. 1020.3820). ^{31}P NMR ($CDCl_3$) δ 149.4, 149.0.



2Y **3Y**

2'-Deoxy-5-(1-(pyrenmethyl)-1*H*-1,2,3-triazol-4-yl)uridine phosphoramidite (3Y). Nucleoside **2Y** (0.20 g, 0.29 mmol) was coevaporated in anh. 1,2-dicholoroethane (3×10mL) and redissolved in anh. CH_2Cl_2 (4.0 mL). To this solution was dropwise added DIPEA (170 μL, 0.99 mmol) and 2-cyanoethyldiisopropylchlorophosporamidite (830 μL, 0.37 mmol) and stirred at rt for 2 h, whereupon the solvent was evaporated under reduced pressure, and the resulting crude was purified via column chromatography (0–3% MeOH in CH_2Cl_2, v/v) to afford amidite **3Y** (0.12g, 66%) as a slightly yellow solid. R_f = 0.6 (5% MeOH in CH_2Cl_2, v/v); MALDI-HRMS *m/z* 1012.4142 ($[M+H]^+$, $C_{58}H_{58}N_7O_8P$ H^+ calc. 1012.4157). ^{31}P NMR ($CDCl_3$) δ 149.4, 148.9.

Oligonucleotide synthesis: Oligonucleotides (ONs) were synthesized on a 0.2 μmol scale performed on an Expedite 8909 Synthesizer using succinyl linked LCAA-CPG (long chain alkyl amine controlled pore glass) columns with a pore size of 500 Å. Standard procedures were used, i.e., trichloroacetic acid in CH_2Cl_2 as detritytilation reagent; 0.25 M 4,5-dicyanoimidazole (DCI) in CH_3CN as activator; acetic anhydride in THF as cap A solution; 1-methylimidazole in THF/pyridine (8:1, v/v) as cap B solution, and 0.02 M iodine in H_2O/pyridine/THF as the oxidizing solution. C5-Pyrene-functionalized triazole-linked phosphoramidtes were incorporated using DCI as the activator and extended coupling times (15 min) which resulted in coupling yields above 98%. Cleavage from the solid support and removal of protecting groups was accomplished by treatment with concentrated aq. ammonia (55 ^{o}C, 24 h). Purification of all modified ONs was performed to minimum 80% purity using either of two methods: (a) overall synthesis yield >80%: cleavage of DMT using 80% aq. AcOH, followed by precipitation from acetone (-18 ^{o}C for 12-16 h) and washing with acetone, or (b) overall synthesis <80%: purification of ONs by RP-HPLC as described below (Table S1), followed by detritylation and precipitation as outlined under (a).

Purification of crude ONs was performed on a Varian Prostar HPLC system equipped with an XTerra MS C18 column (10 μm, 7.8 x 150 mm) using the representative gradient protocol depicted in Table S1. The composition of all synthesized ONs was verified by MALDI MS/MS analysis (Table S2) using anthranilic acid as a matrix and recording in positive ions mode on a Quadropole Time-Of-Flight tandem Mass Spectrometer (Q-TOF Premiere) equipped with a MALDI source (Waters Micromass LTD., U.K.). Purity (>80%) was verified by RP-HPLC.

Table S1. Representative RP-HPLC gradient protocol.[a]

T (min)	Buffer A (V%)	Buffer B (V%)
0	100	0
2	100	0
50	30	70
64	0	100
69	0	100
71	100	0
80	100	0

[a] Buffer A is 0.05 M TEAA (triethyl ammonium acetate) pH 7, while buffer B is 75% MeCN in H_2O v/v. A flow rate of 1.2 mL/min was used.

Table S2. MALDI-MS of synthesized ONs.[a]

ON	Sequences	Calcd *m/z* $[M]^+$	Found *m/z* $[M]^+$
ON5	5'-CGC AAA **X**AA ACG C	4187.8	4188.4
ON6	5'-CGC AAC **X**CA ACG C	4139.8	4140.3
ON7	5'-CGC AAG **X**GA ACG C	4219.8	4220.3
ON8	5'-CGC AAT **X**TA ACG C	4169.8	4170.3
ON9	5'-CGC AAA **Y**AA ACG C	4201.8	4202.4
ON10	5'-CGC AAC **Y**CA ACG C	4153.8	4154.4
ON11	5'-CGC AAG **Y**GA ACG C	4233.8	4234.4
ON12	5'-CGC AAT **Y**TA ACG C	4183.8	4184.4

[a] For structures of monomer **X** and **Y** see Scheme 1 in the main manuscript.

Protocol for thermal denaturation studies: Concentrations of unmodified ONs were estimated using the following extinctions coefficients for DNA (OD/μmol): G (12.01), A (15.20), T (8.40), C (7.05); for RNA (OD/μmol): G (13.70), A (15.40), U (10.00), C (9.00). Concentrations of modified ONs (**ON5-ON12**) were determined by titration of modified ONs against complementary DNA; continued addition of complementary DNA target to **ON5**-**ON12** resulted in increased steady-state fluorescence emission output until a plateau was reached, at which point a 1:1 stoichiometry was assumed. ONs (1.0 μmol of each strand) were thoroughly mixed, denatured by heating and subsequently cooled to the starting temperature of the experiment. Quartz optical cells with a pathlength of 1.0 cm were used. Thermal denaturation temperatures (T_m values [^{o}C]) were measured on a Cary 100 UV/VIS spectrophotometer equipped with 12-cell Peltier temperature controller and determined as the maximum of the first derivative of the thermal denaturation curve (A_{260} vs. T) recorded in medium salt buffer (T_m buffer: 110 mM NaCl, 0.1 mM EDTA, pH adjusted with 10 mM Na_2HPO_4/ NaH_2PO_4). The temperature of the denaturation experiment ranged from at least 15 ^{o}C below T_m to 20 ^{o}C above T_m (although not below 1 ^{o}C). A temperature ramp of 0.5 ^{o}C/min was used in all experiments. Reported thermal denaturation temperatures are an average of at least two experiments within ±1.0 ^{o}C.

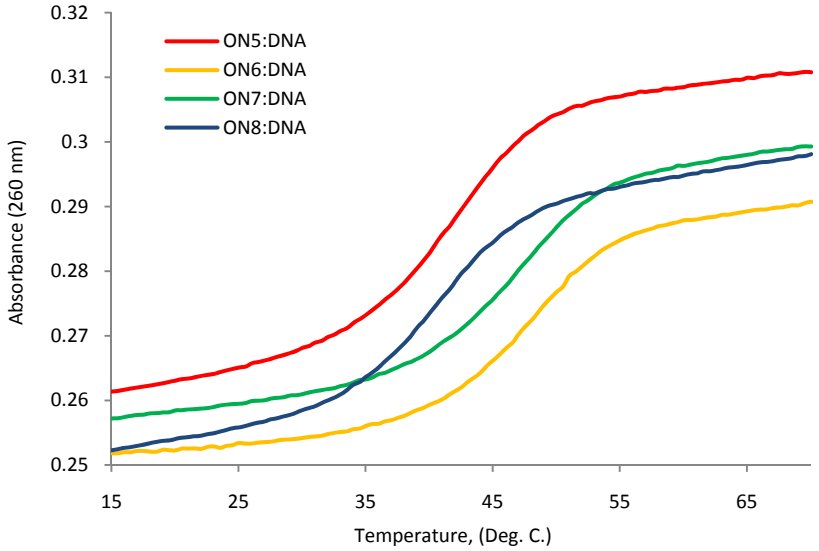


Figure S1. Thermal denaturation curves for duplexes between **ON5**-**ON8** (monomer **X**) and complementary DNA.

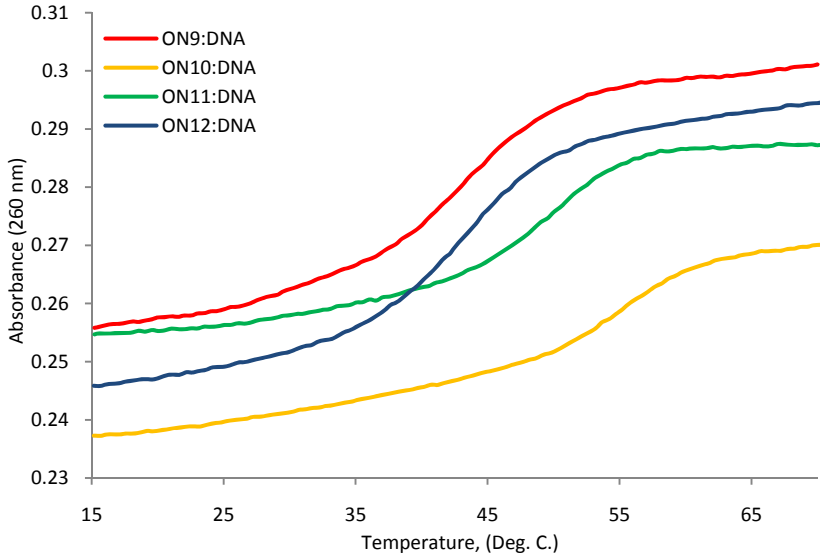


Figure S2. Thermal denaturation curves for duplexes between **ON9**-**ON12** (monomer **Y**) and complementary DNA.

Protocol for fluorescence studies: Steady state fluorescence emission spectra were recorded using an excitation wavelength of 344 nm on a Cary Eclipse fluorimeter using the same buffers and ON concentrations as in thermal denaturation studies and. Fluorescence emission spectra of single stranded probes (SSP) and corresponding duplexes with complementary or mismatched targets were measured at 5 ^{o}C to ensure complete hybridization to targets. Deoxygenation was deliberately not applied to the samples since the scope of the work was to determine fluorescence under aerated condition prevailing in bioassays. Solutions were heated to 80 ^{o}C over 10 min, cooled to 5 ^{o}C over 15 min, and equilibrated at this temperature for more than 5 min. Steady state fluorescence emission spectra (360–600 nm range) were obtained as an average of five scans using an excitation wavelength of 344 nm, excitation slit 5.0 nm, emission slit 5.0 nm and a scan speed of 600 nm/min.

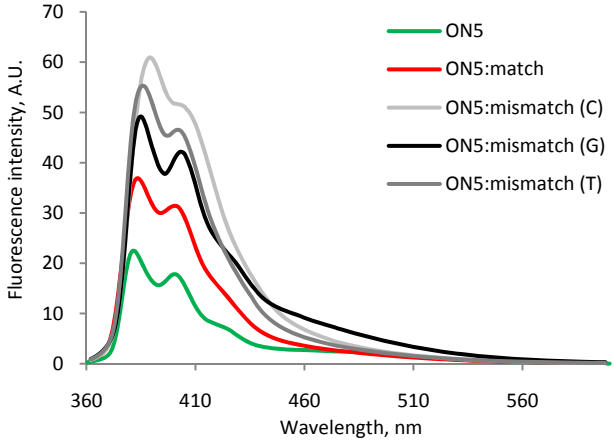


Figure S3. Steady state fluorescence emission spectra of single stranded **ON5** (SSP), and corresponding duplexes with matched or mismatched DNA targets (mismatched nucleotide opposite of modification is mentioned in parenthesis).

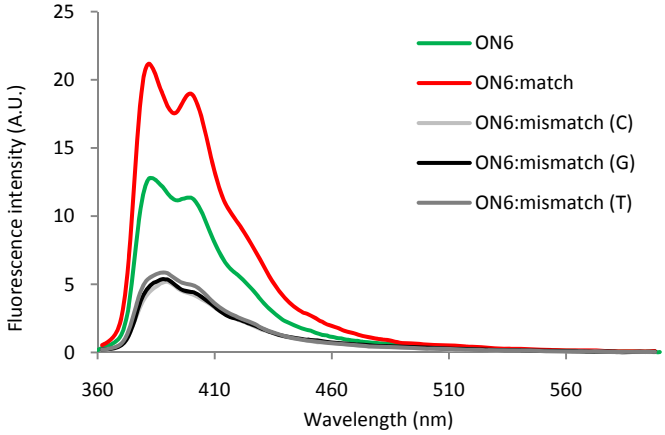


Figure S4. Steady state fluorescence emission spectra of single stranded **ON6** (SSP), and corresponding duplexes with matched or mismatched DNA targets (mismatched nucleotide opposite of modification is mentioned in parenthesis).

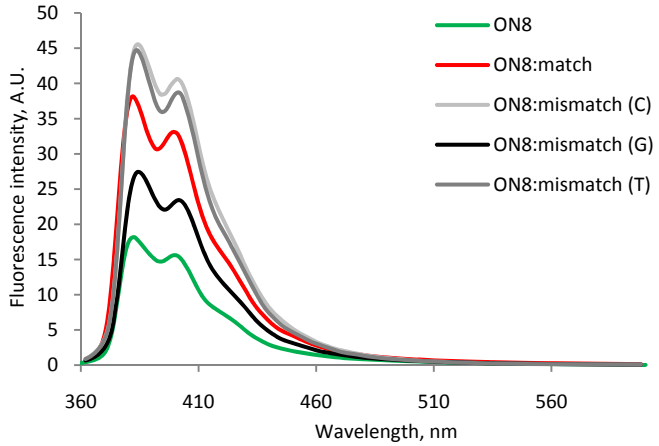


Figure S5. Steady state fluorescence emission spectra of single stranded **ON8** (SSP), and corresponding duplexes with matched or mismatched DNA targets (mismatched nucleotide opposite of modification is mentioned in parenthesis).

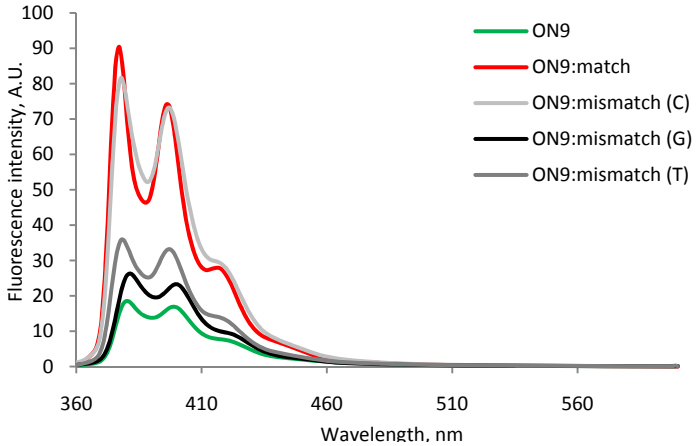


Figure S6. Steady state fluorescence emission spectra of single stranded **ON9** (SSP), and corresponding duplexes with matched or mismatched DNA targets (mismatched nucleotide opposite of modification is mentioned in parenthesis).

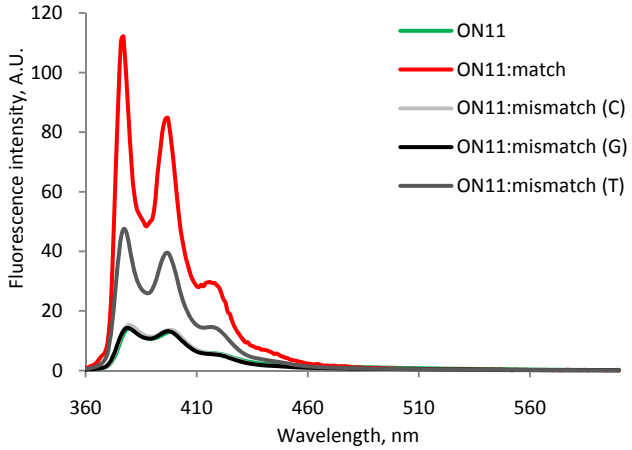


Figure S7. Steady state fluorescence emission spectra of single stranded **ON11** (SSP), and corresponding duplexes with matched or mismatched DNA targets (mismatched nucleotide opposite of modification is mentioned in parenthesis).

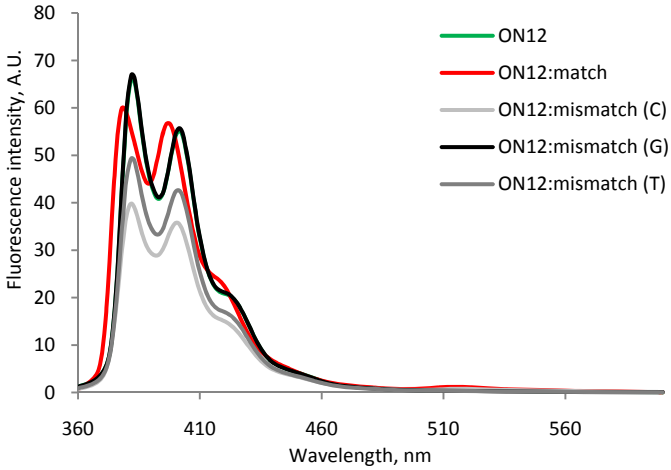


Figure S8. Steady state fluorescence emission spectra of single stranded **ON12** (SSP), and corresponding duplexes with matched or mismatched DNA targets (mismatched nucleotide opposite of modification is mentioned in parenthesis).

Table S3. Thermal denaturation temperatures of duplexes between **ON2, ON6** or **ON10** and complementary or mismatched RNA targets.[a]

		3'-r(GCG UUG **B**GU UGC G)			
		ΔT_m/mod. [°C]	ΔT_m [°C]		
	Sequence	**B**=A	**B**=C	**B**=G	**B**=U
ON2	5'-CG CAA CTC AAC GC	**51.5**	36.0 (-15.5)	48.5 (-3.0)	38.0 (-13.5)
ON6	5'-CG CAA C**X**C AAC GC	**39.5** (-12.0)	32.0 (-7.5)	36.0 (-3.5)	33.0 (-6.5)
ON10	5'-CG CAA C**Y**C AAC GC	**48.0** (-3.5)	29.5 (-18.5)	41.0 (-7.0)	35.0 (-13.0)

[a] Conditions as described in footnote of Table 1.

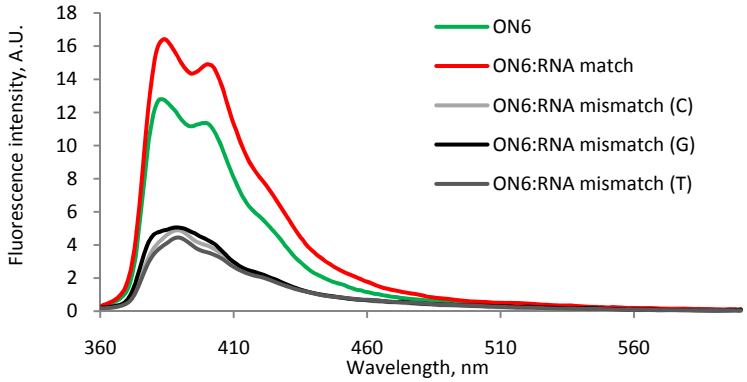


Figure S9. Steady state fluorescence emission spectra of single stranded **ON6** (SSP), and corresponding duplexes with matched or mismatched RNA targets (mismatched nucleotide opposite of modification is mentioned in parenthesis).

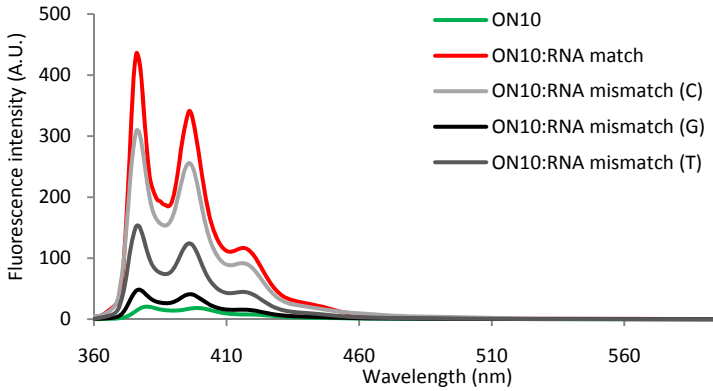
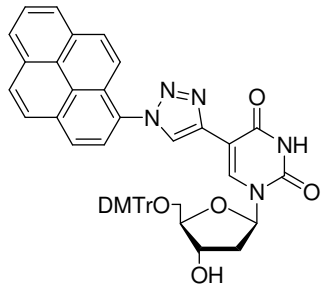
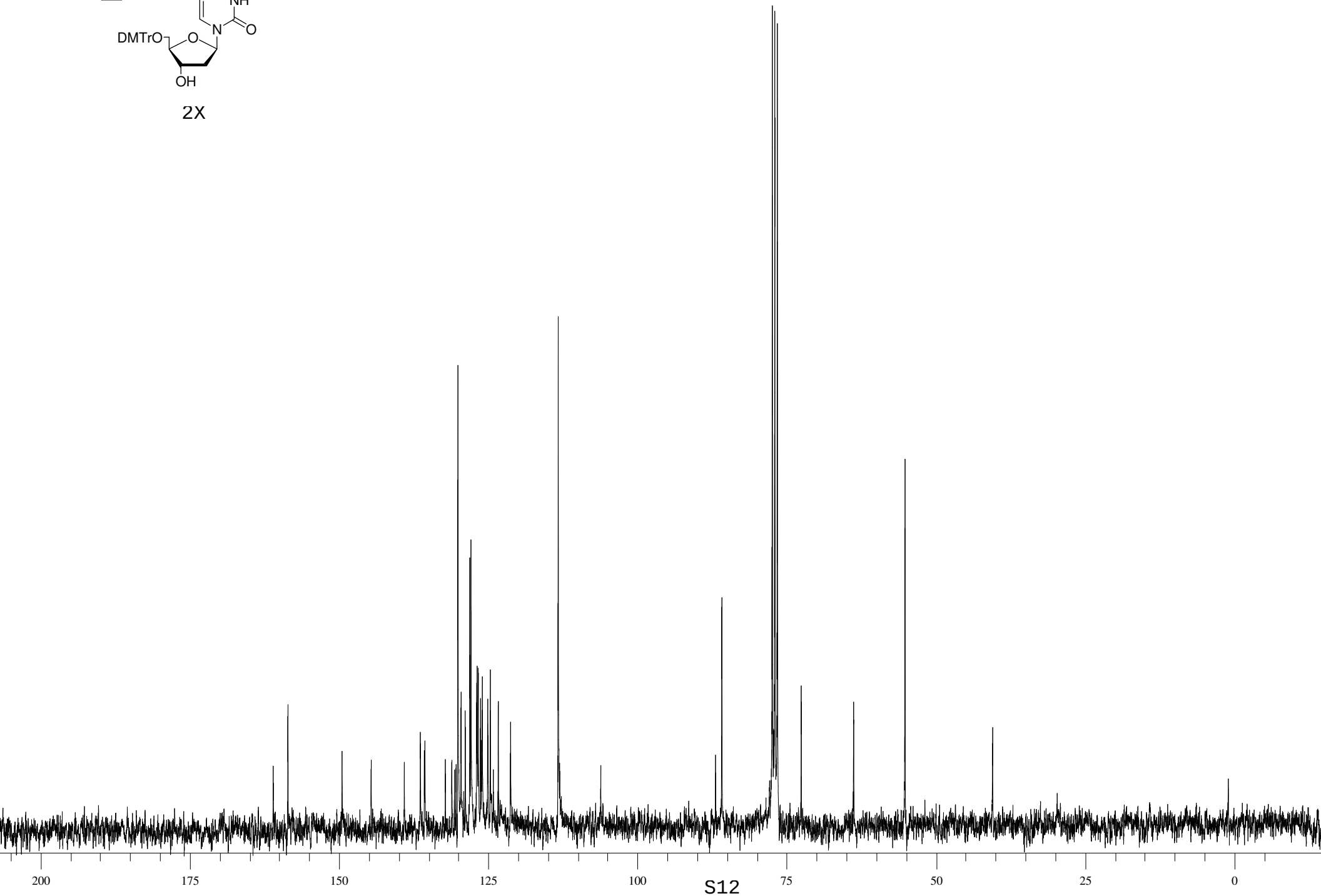
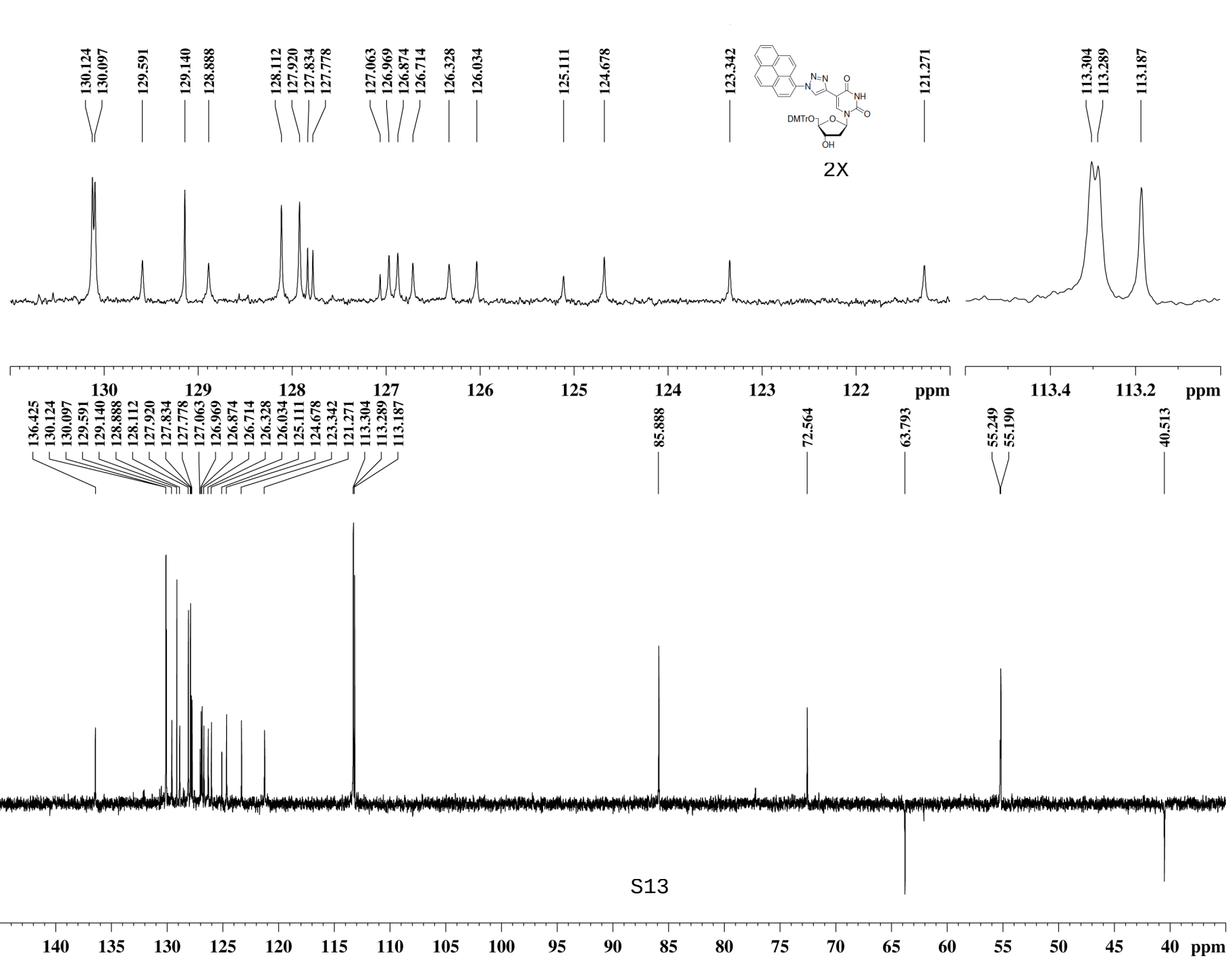


Figure S10. Steady state fluorescence emission spectra of single stranded **ON10** (SSP), and corresponding duplexes with matched or mismatched RNA targets (mismatched nucleotide opposite of modification is mentioned in parenthesis).



2X

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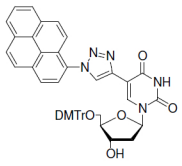
129.591
129.140
128.888
128.112
127.920
127.834
127.778
127.063
126.969
126.874
126.714
126.328
126.034
125.111
124.678
123.342
121.271
113.304
113.289
113.187
85.888
72.564
63.793
55.249
55.190
40.513
S13
140
135
130
125
120
115
110
105
100
95
90
85
80
75
70
65
60
55
50
45
40
ppm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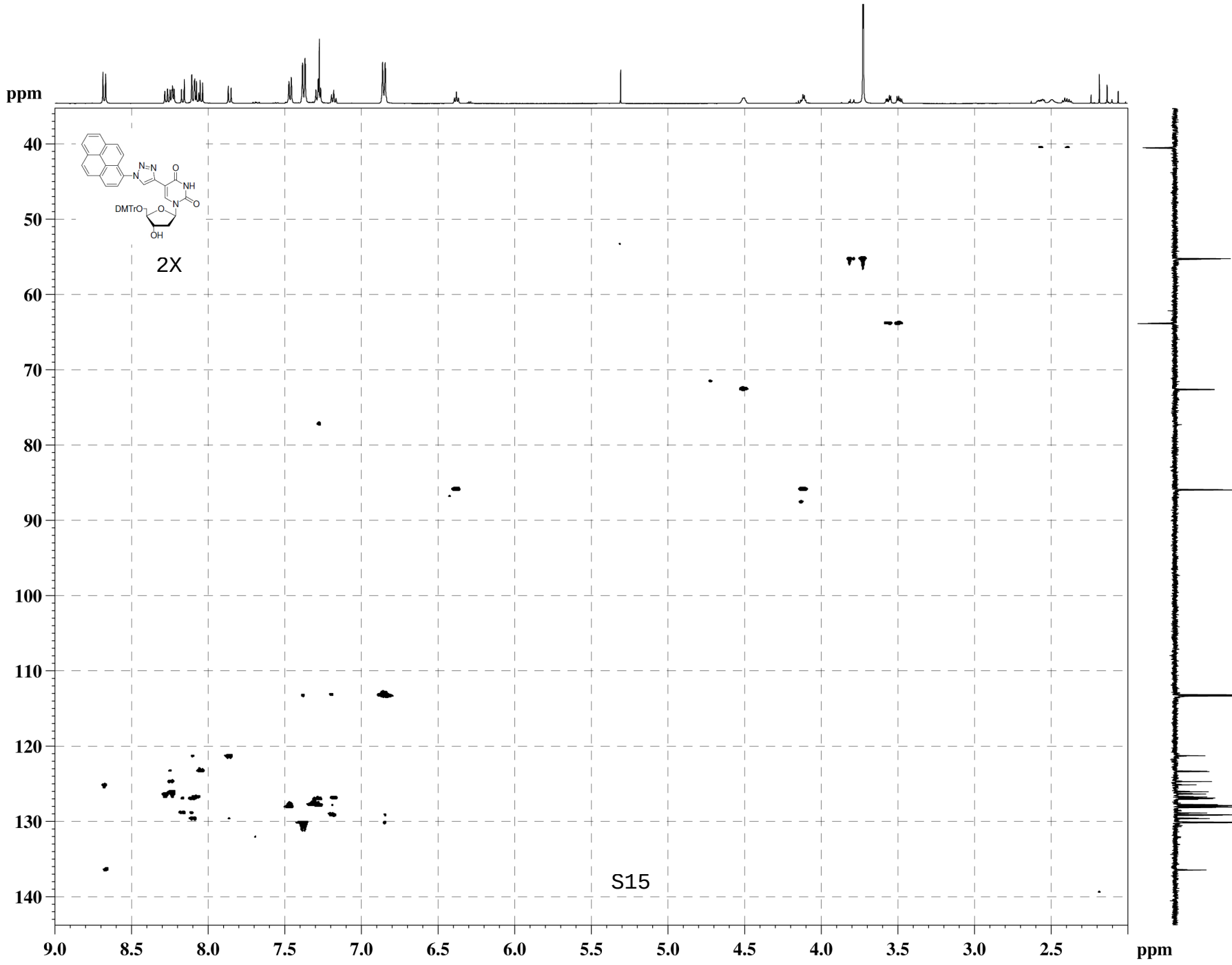
O
NH
O
N
DMTrO
O
OH

2X

ppm
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.0
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
ppm


ppm
40
50
60
70
80
90
100
110
120
130
140
9.0
8.5
8.0
7.5
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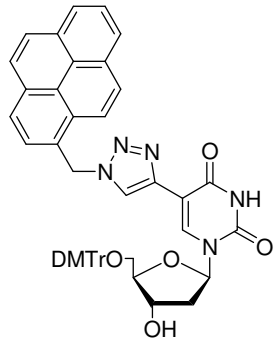
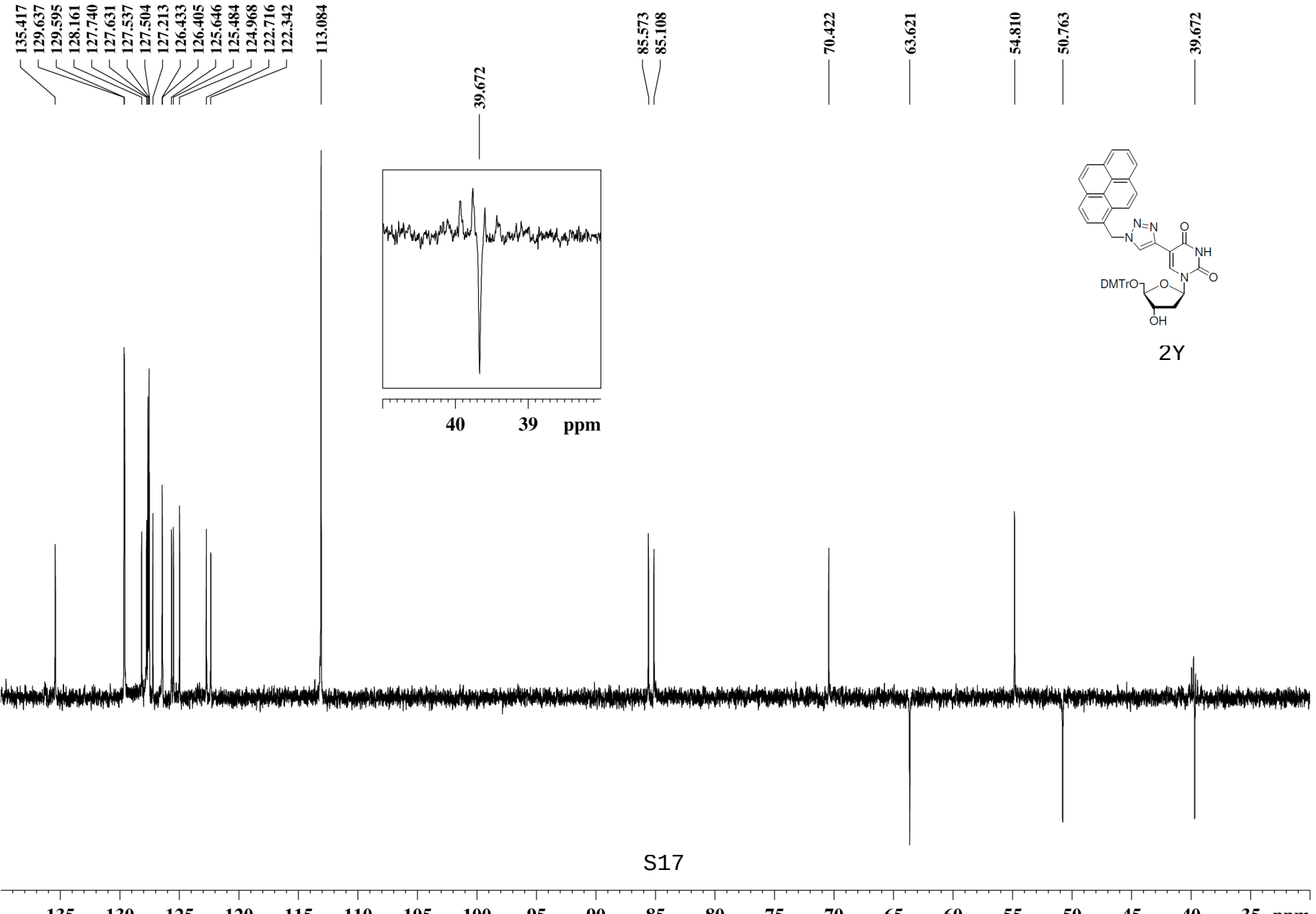
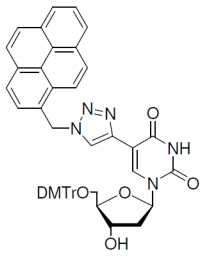
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
ppm
DMTrO
OH
NH
O
N
2X
S15



2Y


135.417
129.637
129.595
128.161
127.740
127.631
127.537
127.504
127.213
126.433
126.405
125.646
125.484
124.968
122.716
122.342
113.084
39.672
85.573
85.108
70.422
63.621
54.810
50.763
39.672
40
39
ppm
DMTrO
O
N
N
N
NH
O
O
OH
2Y
S17
135
130
125
120
115
110
105
100
95
90
85
80
75
70
65
60
55
50
45
40
35
ppm

]


N
N
N
O
NH
O
DMTrO
O
N
OH

2Y

ppm
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
8.5
8.0
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7.0
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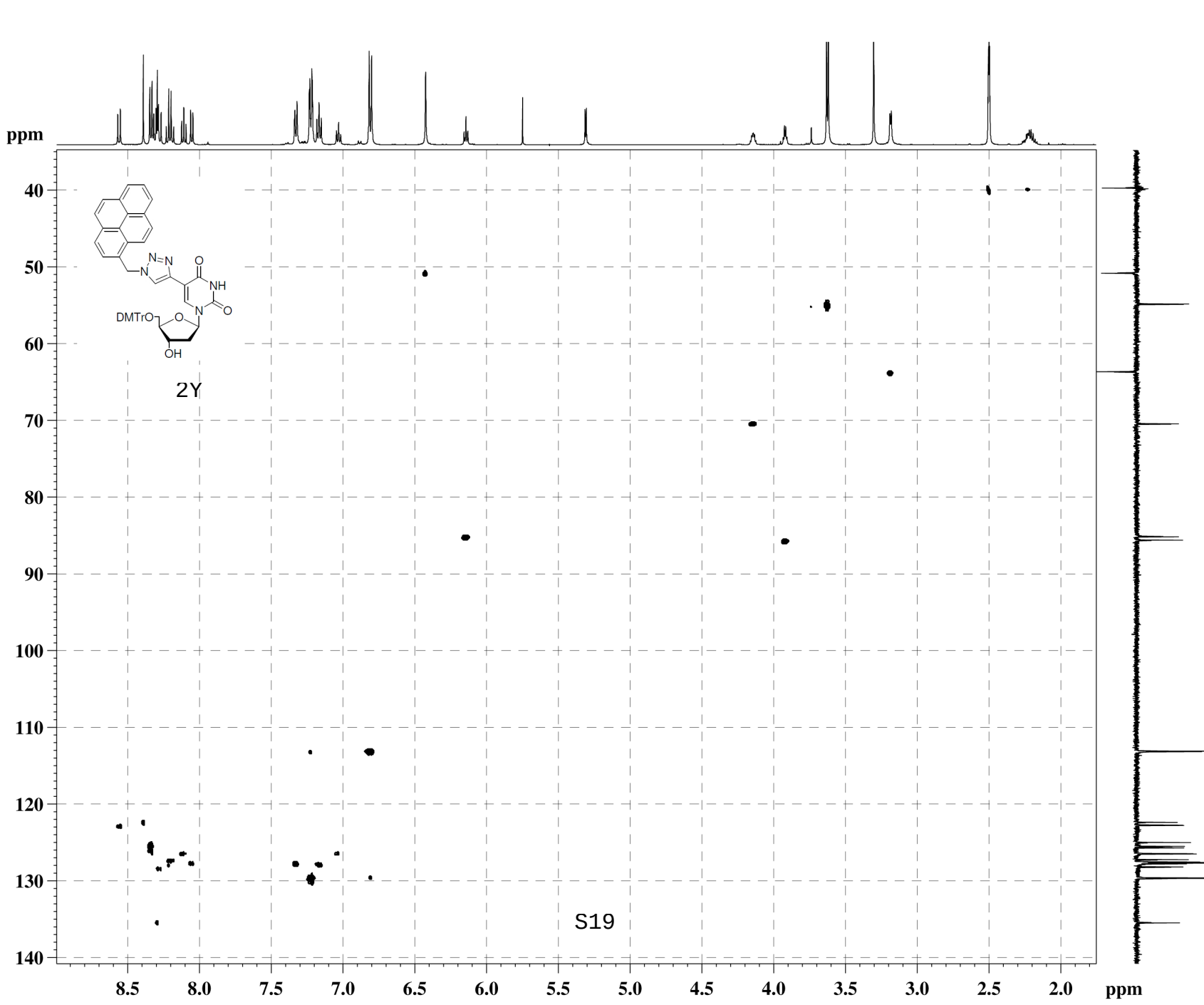
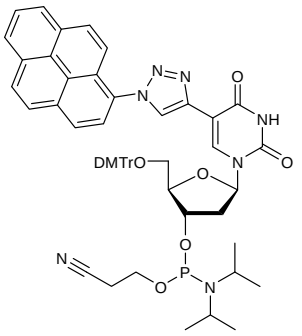
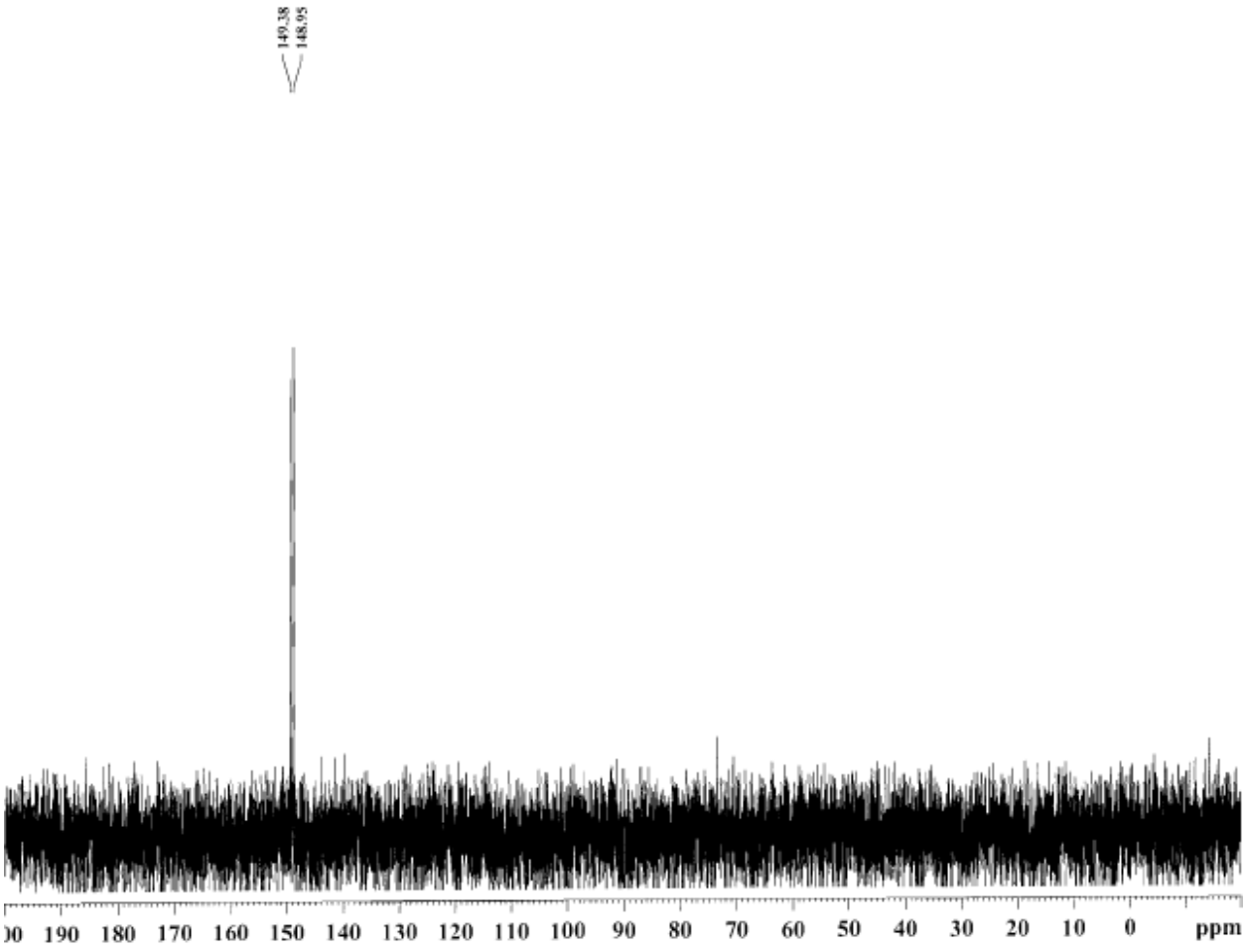
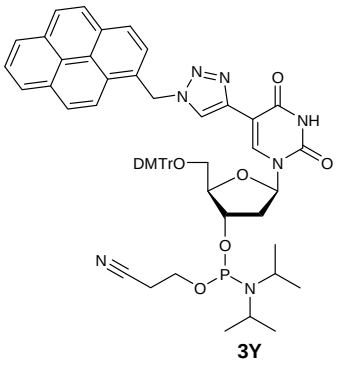
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
ppm


ppm
40
50
60
70
80
90
100
110
120
130
140
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
ppm
DMTrO
O
NH
N
N
N
O
O
OH
2Y
S19


N
N
N
O
NH
O
N
DMTrO
O
O
N
P
O
N

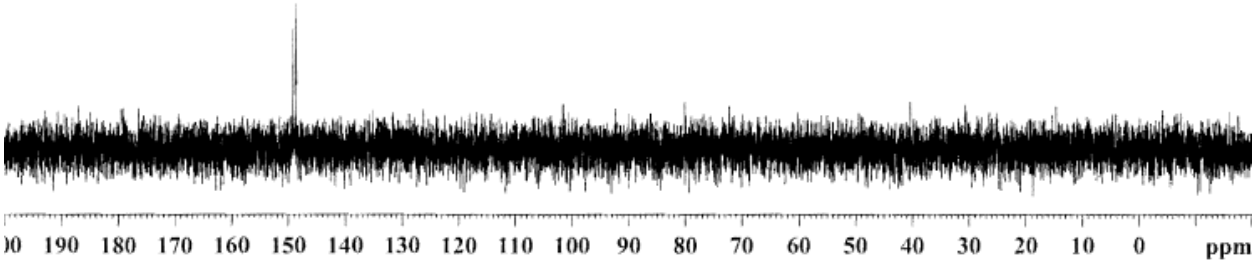
3X


149.38
148.95
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm


N
N
N
O
NH
O
N
DMTrO
O
O
N
O
P
O
N

3Y


149.35
148.87


190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm