Post-synthesis DNA Modifications Using a *trans*-Cyclooctene Click Handle

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Supporting Information

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1. General Information

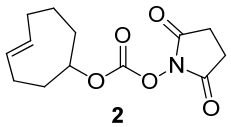
Solvents and reagents were purchased from VWR International, Oakwood Product Inc., or Sigma-Aldrich Co. and used without purification unless specified otherwise. When necessary, solid reagents were dried under high vacuum. Reactions with compounds sensitive to air or moisture were performed under argon. Solvent mixtures are indicated as volume/volume ratios. Thin layer chromatography (TLC) was run on Sorbtech W/UV254 plates (0.25 mm thick), and visualized under UV-light. Flash chromatography was performed using Fluka silica gel 60 (mesh size: 0.040-0.063 mm) using a weight ratio of ca. 30:1 for silica gel over crude compound. ^{1}H, ^{13}C, and ^{31}P-NMR spectra were recorded on a Bruker Avance 400 spectrometer (400, 100, and 166 MHz, respectively) in deuterated chloroform ($CDCl_3$), methanol-d_4 (CD_3OD), and DMSO-d_6

with either tetramethylsilane (TMS) (0.00 ppm) or the NMR solvent as the internal reference. HPLC purification for *trans*-cyclooctene triphosphate (TCO-TTP) was carried out using a Shimadzu LC-20AT VP system with a Zobax C18 reversed-phase column (9.4 mm × 25 cm). The sample was eluted (3 mL/min) with buffer A (20 mM triethylammonium acetate, pH 6.9-7.1) and buffer B (50% acetonitrile, 20 mM triethyl ammonium acetate) with the following: 0 min 0% B; 20 min 20% B; 30 min 100% B; 38 min 100% B; 40 min 0% B; and 45 min 0% B.

DNA primers and templates were purchased from Integrated DNA Technologies. Klenow fragment (3'-5' exo), *Taq.* DNA polymerase and Deep Vent$_R$ (exo-) DNA polymerase were purchased from New England Biolabs. KOD-XL DNA polymerase was purchased from EMD Chemicals. Reaction buffers were used as provided.

2. Experimental Procedure for the Synthesis of TCO-TTP

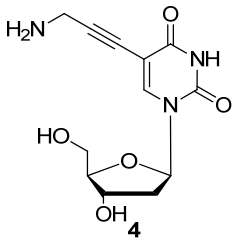
(*E*)-Cyclooct-4-en-1-yl (2,5-dioxopyrrolidin-1-yl) carbonate **2**



2

Compound **2** was prepared starting from cyclooctadiene (**1**) in four steps following literature procedures.[1]

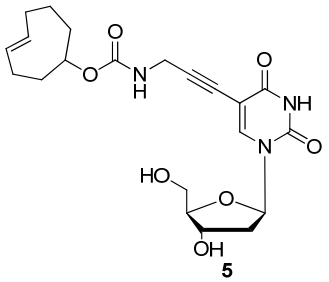
5-(3-Aminoprop-1-yn-1-yl)-1-((2R,4S,5R)-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione **4**



4

Compound **4** was synthesized starting from 5-odo-2'-deoxyuridine (**3**) in two steps following literature procedures.[2, 3]

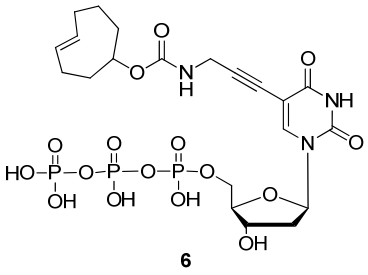
(*E*)-Cyclooct-4-en-1-yl(3-(1-((2R,4S,5R)-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl) 2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)prop-2-yn-1-yl)carbamate **5**



5

To a mixture of succimidyl ester **2** (134 mg, 0.5 mmol) and amine modified deoxyuridine **4** (168 mg, 0.6 mmol) in DMF 10 mL was added DIPEA (129 mg, 1 mmol). The reaction mixture was wrapped in foil and stirred in r.t. for 12 h. Reaction solution was concentrated under reduced pressure and purified with silica gel chromatography (12% MeOH in DCM) to give a white solid (108 mg, yield 52%). ^{1}H NMR (methanol-*d*4): 8.25 (s, 1H), 6.25 (t, *J* = 6.4 Hz, 1H), 5.58-6.20 (m, 1H), 5.47-5.57 (m, 1H), 4.05 (s, 2H), 3.95-3.81 (m, 1H), 3.73-3.77 (m, 2H), 3.24-3.37 (m, 2H), 2.26-2.37 (m, 4H), 2.21-2.25 (m, 1H), 1.99-2.00 (m, 4H), 1.93-1.98 (m, 2H). ^{13}C NMR (methanol-*d*4): 163.1, 149.8, 144.0, 134.7, 132.4, 128.4, 98.6, HRMS (ESI) for $C_{21}H_{27}N_3O_7$ $[M+H]^+$ Cacld: 434.1849; Found: 434.1899.

(*E*)-Cyclooct-4-en-1-yl(3-(1-((2R,4S,5R)-4-hydroxy-5-(((hydroxy((hydroxy(phosphonooxy)phosphoryl)oxy)phosphoryl)oxy)methyl)tetrahydrofuran-2-yl)-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)prop-2-yn-1-yl)carbamate **6**



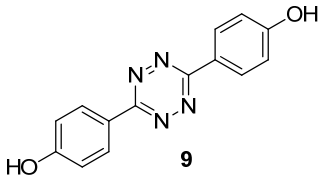
6

To a solution of TCO-T **5** (69 mg, 0.16 mmol) and proton sponge (42 mg, 0.19 mmol, pre-dried in vacuum over P_2O_5 overnight) in anhydrous trimethylphosphate (0.6 mL) was added freshly distilled $POCl_3$ (18 μL, 0.19 mmol) dissolved in anhydrous trimethylphosphate (0.2 mL) dropwise via a syringe with stirring under argon at 0 °C. The reaction mixture was further stirred

in an ice-bath for 2 h and then a solution of tributylammonium pyrophosphate (226 mg, 0.48 mmol) and tri-*n*-butylamine (0.4 mL,) in 1.0 mL of anhydrous DMF was added in one portion. The mixture was stirred at RT for 10 min and then triethylammonium bicarbonate solution (0.1 M, pH 8, 11 mL) was added. After stirring at r.t. for an additional 1 h, the resulting reaction mixture was transferred to a 50-mL centrifuge tube. Then EtOH (33 mL) was added followed by 3 M NaCl solution (1.0 mL). After vortexing for 10 sec, the centrifuge tube was placed at -80 ^{o}C for 1 h, and then centrifuged at 5000 rpm for 20 min. After removing the supernatant, the resulting pellet was purified by HPLC and lyophilized to give a pale white powder (32 mg, 32%). ^{1}H NMR (D_2O): 8.06 (s, 1H), 6.18 (t, J = 6.8 Hz, 1H), 5.58-6.20 (m, 1H), 5.47-5.57 (m, 1H), 4.16-4.25 (m, 2H), 4.08-4.12 (m, 3H), 4.0 (d, J = 7.2 Hz, 1H), 2.25-2.34 (m, 5H), 1.88-1.98 (m, 3H), 1.35-1.86 (m, 4H). ^{13}C NMR (D_2O):179.7, 164.6, 158.0, 150.0, 144.8, 135.6, 133.4, 99.2, 82.4, 73.2, 70.5, 65.3, 46.6, 40.4, 38.7, 37.8, 33.7, 32.0, 30.6, 22.2, 8.2; ^{31}P NMR (D_2O) -10.9, -11.6, -23.3. MS (ESI) for $C_{21}H_{30}N_3O_{16}P_3$ $[M-H]^-$ Cacld. 672.08; Found: 672.1

3. Experimental Procedure for the Synthesis of Tz-BBA

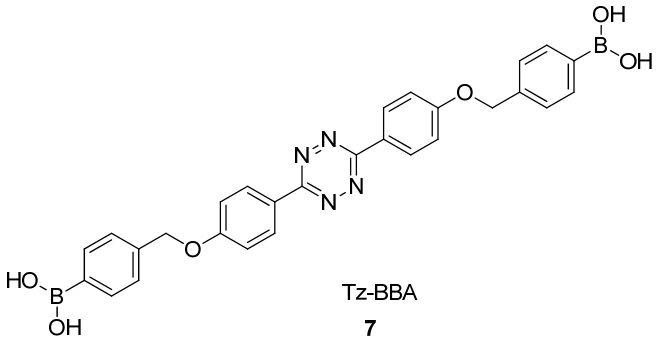
4,4'-(1,2,4,5-tetrazine-3,6-diyl)diphenol **9**



9

Compound **9** was prepared starting from 4-hydroxybenzonitrile in two steps following literature procedures.[4]

Tz-BBA (**7**)



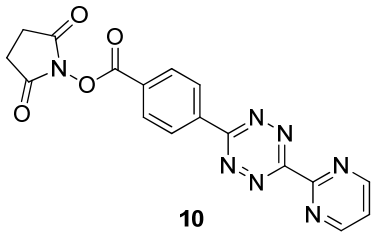
Tz-BBA

7

To a solution of compound **9** (133 mg, 0.5 mmol) was added 4-(bromomethyl)phenylboronic acid (219 mg, 1.3 mmol), $CsCO_3$ (488 mg, 1.5 mmol), and NaI (30 mg, 0.2 mmol) in acetone (20 mL). The mixture was heated to reflux and stirred at 60 ^oC overnight. The solution was concentrated under vacuum and acidified by 1M HCl. The residue was washed with EtOAc and MeOH to afford Tz-BBA **7**. ^1H NMR (DMSO-*d6*): 8.47 (d, *J* = 8 Hz, 4H), 7.83 (d, *J* = 8 Hz, 4H), 7.46 (d, *J* = 8 Hz, 4H), 7.31 (d, *J* = 8 Hz, 4H), and 5.28 (s, 4H). HRMS (ESI) for $C_{28}H_{24}B_2N_4O_6$ $[M+H]^+$ Cacld: 534.1882; Found: 535.1965.

4. Experimental Procedure for the Synthesis of Tz-FITC

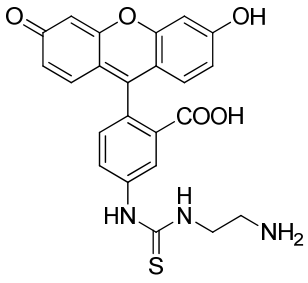
2,5-Dioxopyrrolidin-1-yl 4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzoate **10**



10

Compound **10** was prepared starting from pyrimidine-5-carbonitrile in three steps following literature procedures.[5]

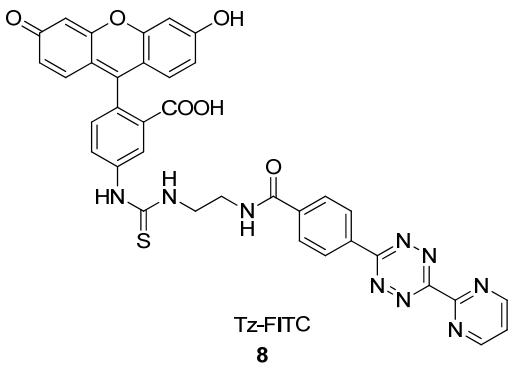
5-(3-(2-Aminoethyl)thioureido)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid **11**



11

Compound **11** was prepared starting from commercially available Fluorescein isothiocyanate (FITC) in one step following literature procedures.[6]

Tz-FITC (**8**)



Tz-FITC

8

To a mixture of compound **10** (188 mg, 0.5 mmol) and compound **11** (112 mg, 0.25 mmol) in MeOH (20 mL) was added Et_3N (55 mg, 0.5 mmol). The mixture was stirred in r.t. overnight. The reaction mixture was acidified to pH 2 and extracted with DCM. Residue was concentrated under vacuo and purified with silica gel chromatography (20% MeOH in DCM) to give product with 40% yield. ^{1}H NMR (DMSO-*d6*): 10.42 (s, 1 H), 9.21 (d, *J* = 4.8, 2 H), 8.97 (s, 1 H), 8.67 (d, *J* = 8.2, 2 H), 8.48 (br, 1 H), 8.30 (s, 1 H), 8.21 (d, *J* = 8.2, 2 H), 7.85 (t, *J* = 4.8, 1 H), 7.80 (d, *J* = 7.9, 1 H), 7.18 (d, *J* = 8.4, 1 H), 6.69 (s, 2 H), 6.59 (m, 4 H), 3.78 (s, 2 H). HRMS (ESI) for $C_{36}H_{25}N_9O_6S$ $[M-H]^-$ Cacld: 710.1549; Found: 710.1589

5. Thermo-stability Study of *trans*-Cyclooctene

5.1 NMR stability test

To investigate *trans*-cyclooctene stability at high temperature, *trans*-cyclooctene **4** sample was dissolved and heated in DMSO-*d6* at 90 ^oC. NMR Spectrum was scanned and recorded every 10 min to observe the isomerization from *trans*-isomer to *cis*-isomer. Isomerization percentage was calculated with peak area at chemical shift δ 5.55. Compared to the *cis*-cycloocetene peak, only 2% and 10% of *trans*-cyclooctene changed to *cis*-isomer after heating at 90 ^oC for 20 min and 70 min (Figure S**1**).

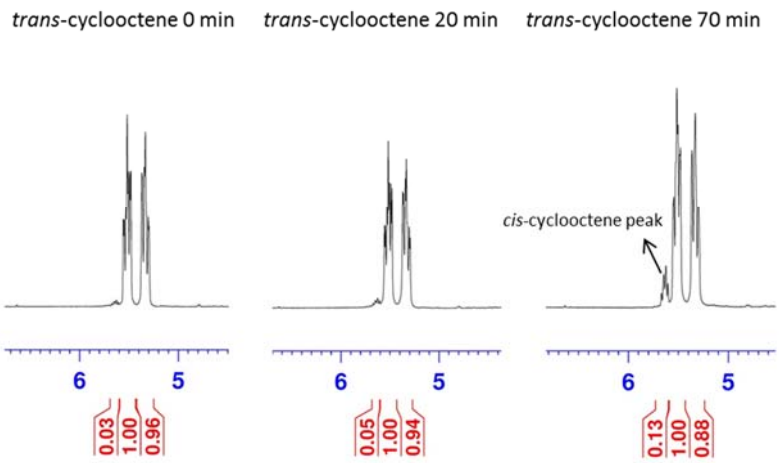
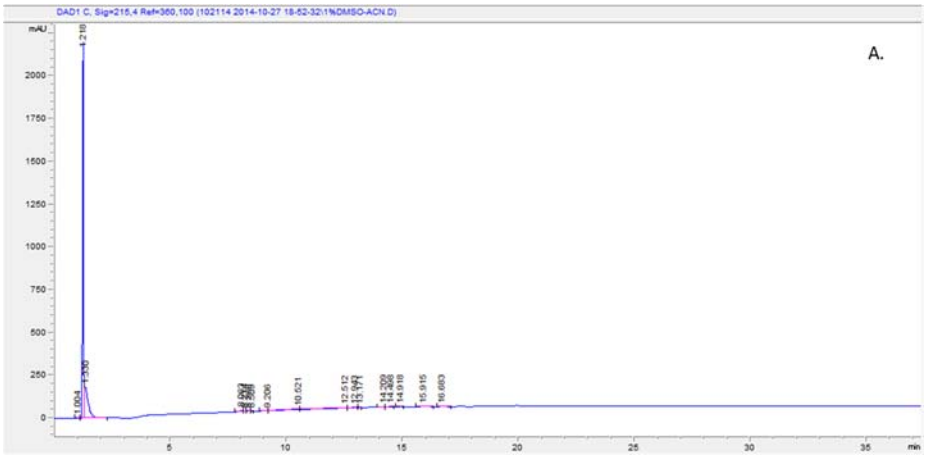


Figure S**1**. NMR study of thermo-stability of *trans*-cyclooctene

5.2 HPLC stability test

In addition to the NMR study, TCO thermo-stability was further examined with HPLC. As shown in Figure S2, there was no noticeable difference after heating the TCO at 90 ºC for 20 min (Figure S2C). After heating at 90 ºC for 70 min, almost 20% of TCO underwent isomerization to give the *cis*-isomer (Figure S2D).



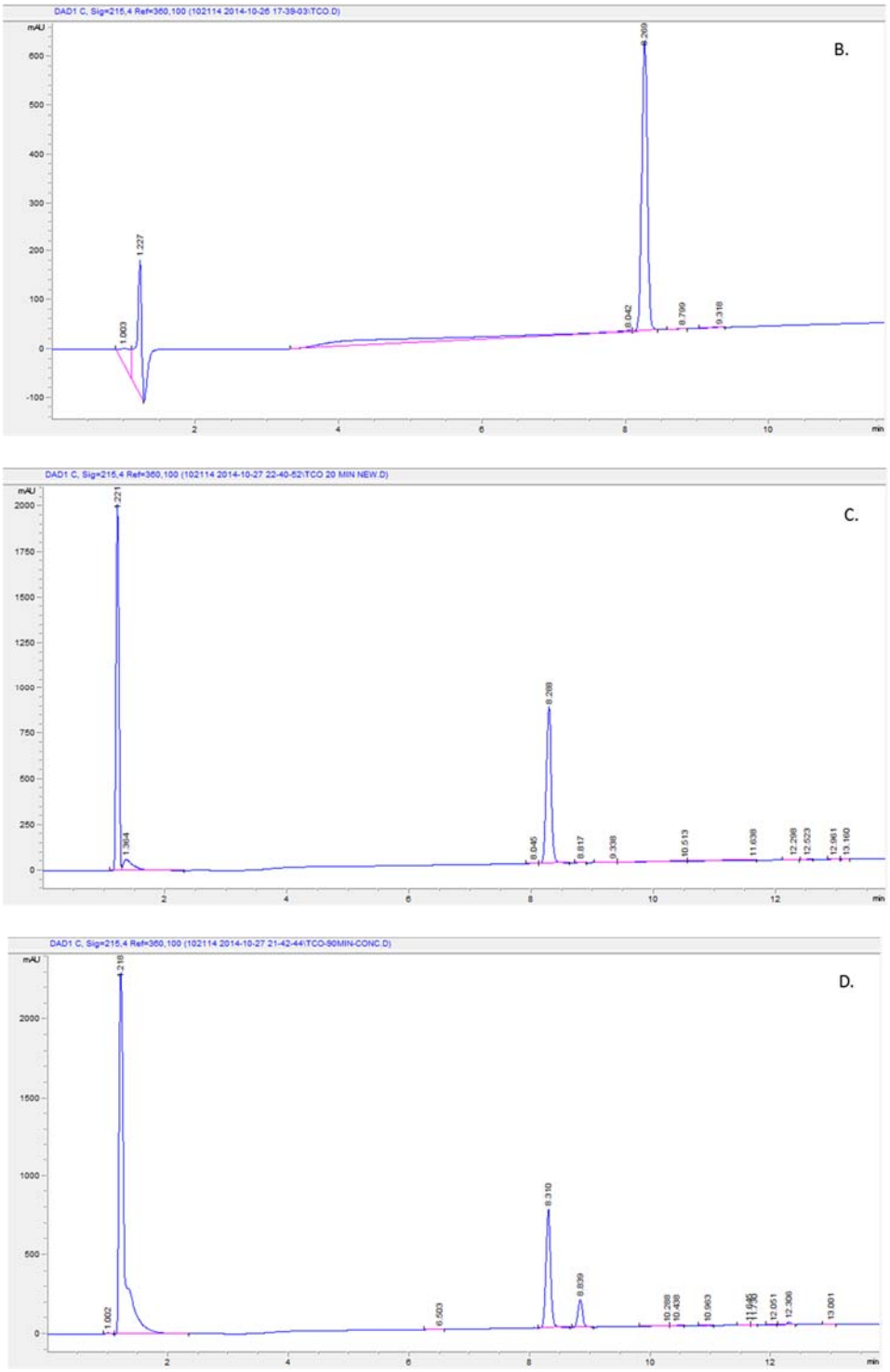


Figure S**2**. HPLC studies of the thermo-stability of *trans*-cyclooctene. A). Background, ACN+ 1%DMSO; B). TCO dissolved in ACN; C). TCO heated at 90 ^{o}C for 20 min; D). TCO heated at 90 ^{o}C for 70 min

6. MALDI-TOF Spectra of DNA Products

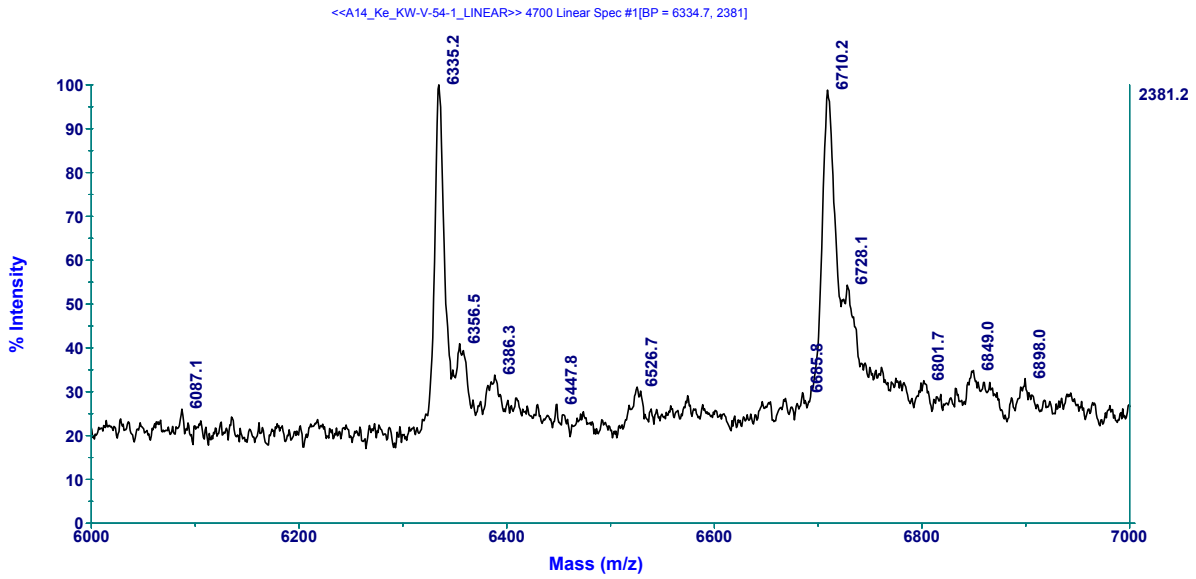


Figure S**3**. MALDI-TOF spectrum of TCO-DNA_{21} (Template-1)

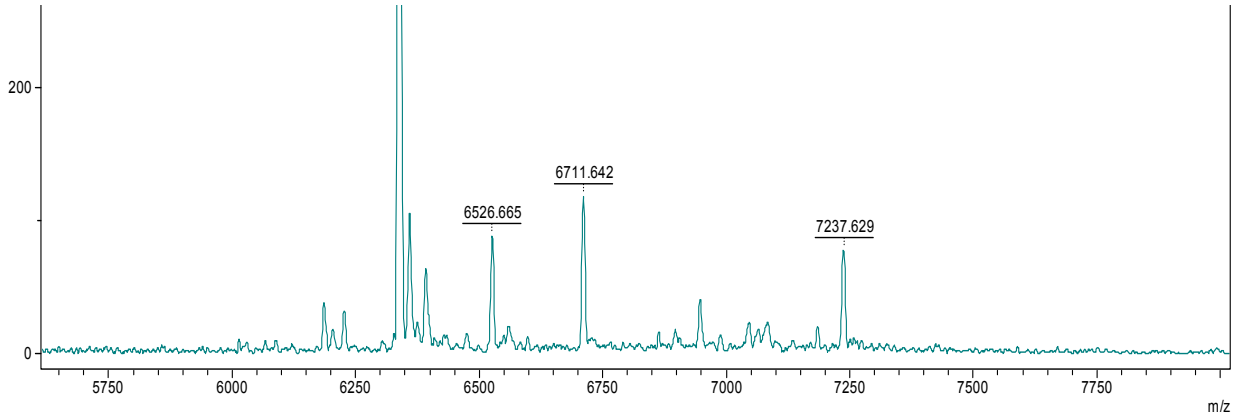


Figure S**4**. MALDI-TOF spectrum of TCO-DNA_{21} (Template-1) after reacting with Tz-BBA

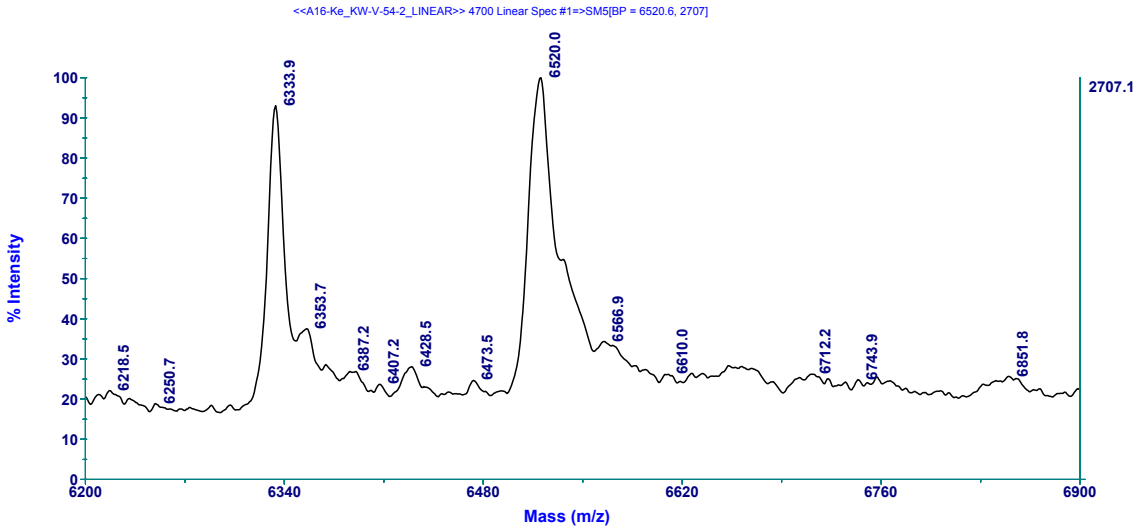
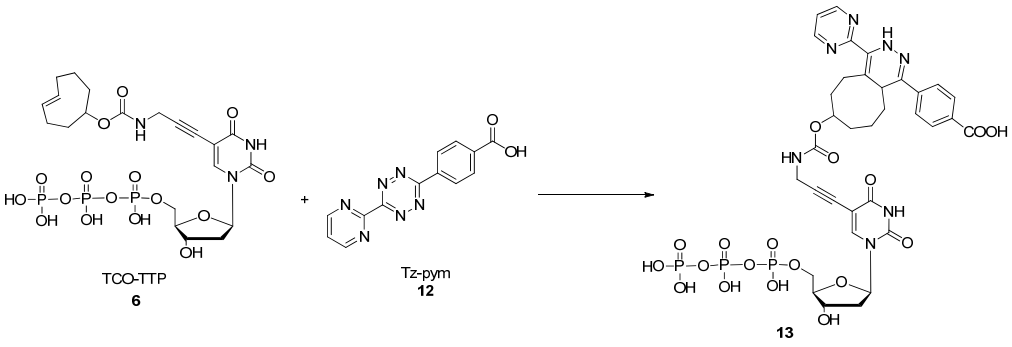


Figure S**5**. MALDI-TOF spectrum of dNTP-DNA_{21} (Template-1) after reacting with Tz-BBA

7. Studies of the reaction kinetics between TCO-TTP and Tetrazine

7.1 Study of reaction kinetics between TCO-TTP and 4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl) benzoic acid

To a solution of TCO-TTP in H_2O was added a solution of 4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzoic acid (**12**, Tz-pym, core structure of Tz-FITC) aqueous solution to afford a final concentration of 20, 25, 30, and 40 μM for TCO-TTP, and a final concentration of 2 μM for Tz-pym. The resulting mixture was examined by monitoring its absorption at 327 nm. Using established procedures,[7] the second-order rate constant was determined to be 1087 $M^{-1}s^{-1}$.



Scheme S**1**. Reaction scheme of TCO-TTP and Tz-pym

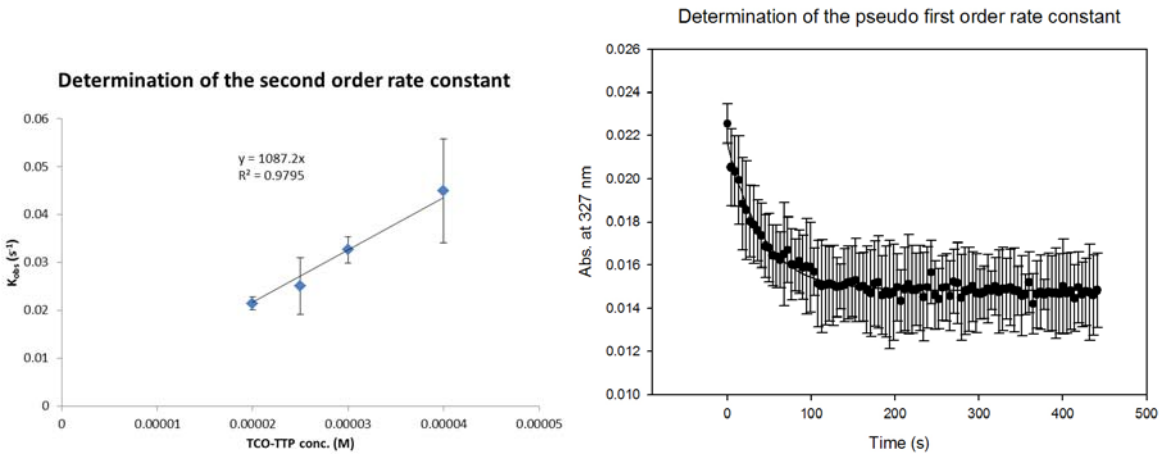
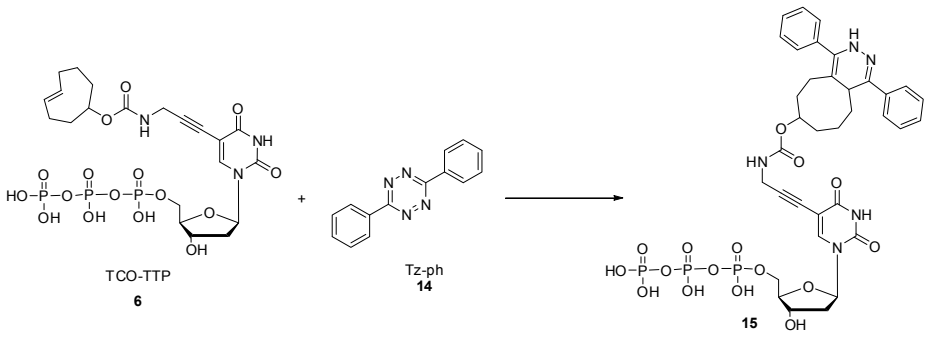


Figure S**6**. Left: Determination of the second order rate constant for the reaction in Scheme S**1**: TCO-TTP final concentration: 20, 25, 30, 40 μM; Tz-pym final concentration: 2 μM. Second order rate constant K_2= 1087 $M^{-1}{\cdot}s^{-1}$. Right: Determination of the pseudo first order rate constant at 25 μM TCO-TTP. Data represents the average of three independent experiments.

7.2 Study of reaction kinetics between TCO-TTP and 3,6-diphenyl-1,2,4,5-tetrazine

To a solution of TCO-TTP in H_2O was added a solution of 3,6-diphenyl-1,2,4,5-tetrazine (**14**, Tz-ph, core structure of Tz-BBA) aqueous solution to afford a final concentration of 30, 40, 50 and 60 μM for TCO-TTP, and a final concentration of 3 μM for Tz-ph. The resulting mixture was examined by monitoring its absorption at 391 nm. Using established procedures,[7] the second-order rate constant was determined to be 27 $M^{-1}{\cdot}s^{-1}$.



Scheme S**2.** Reaction scheme of TCO-TTP and Tz-ph

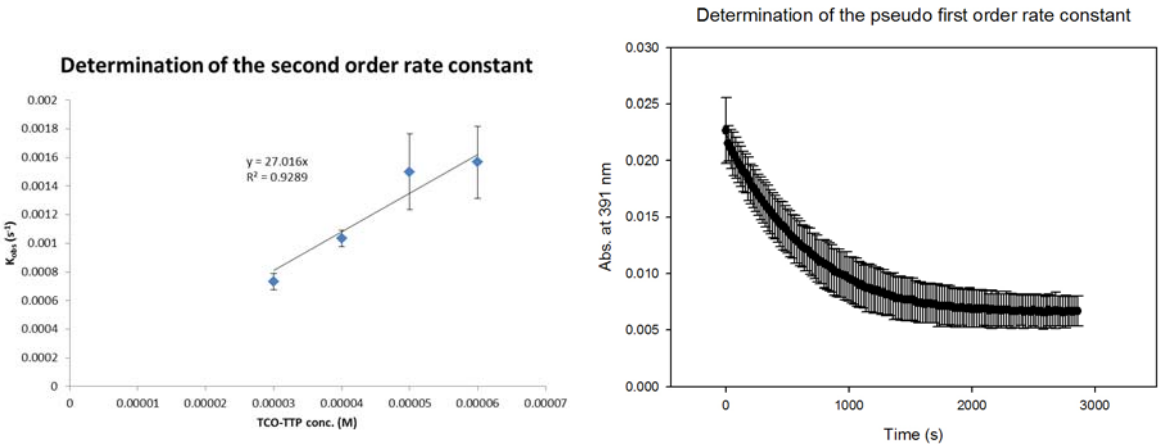


Figure S**7**. Left: Determination of the second order rate constant for the reaction in Scheme S**2**: TCO-TTP final concentration: 30, 40, 50, 60 μM; Tz-ph final concentration: 3 μM. Second order rate constant K_2= 27 $M^{-1} \cdot s^{-1}$. Right: Determination of the pseudo first order rate constant at 60 μM TCO-TTP. Data represents the average of three independent experiments.

8. NMR Spectra of Synthesized Compounds (Please see next pages)

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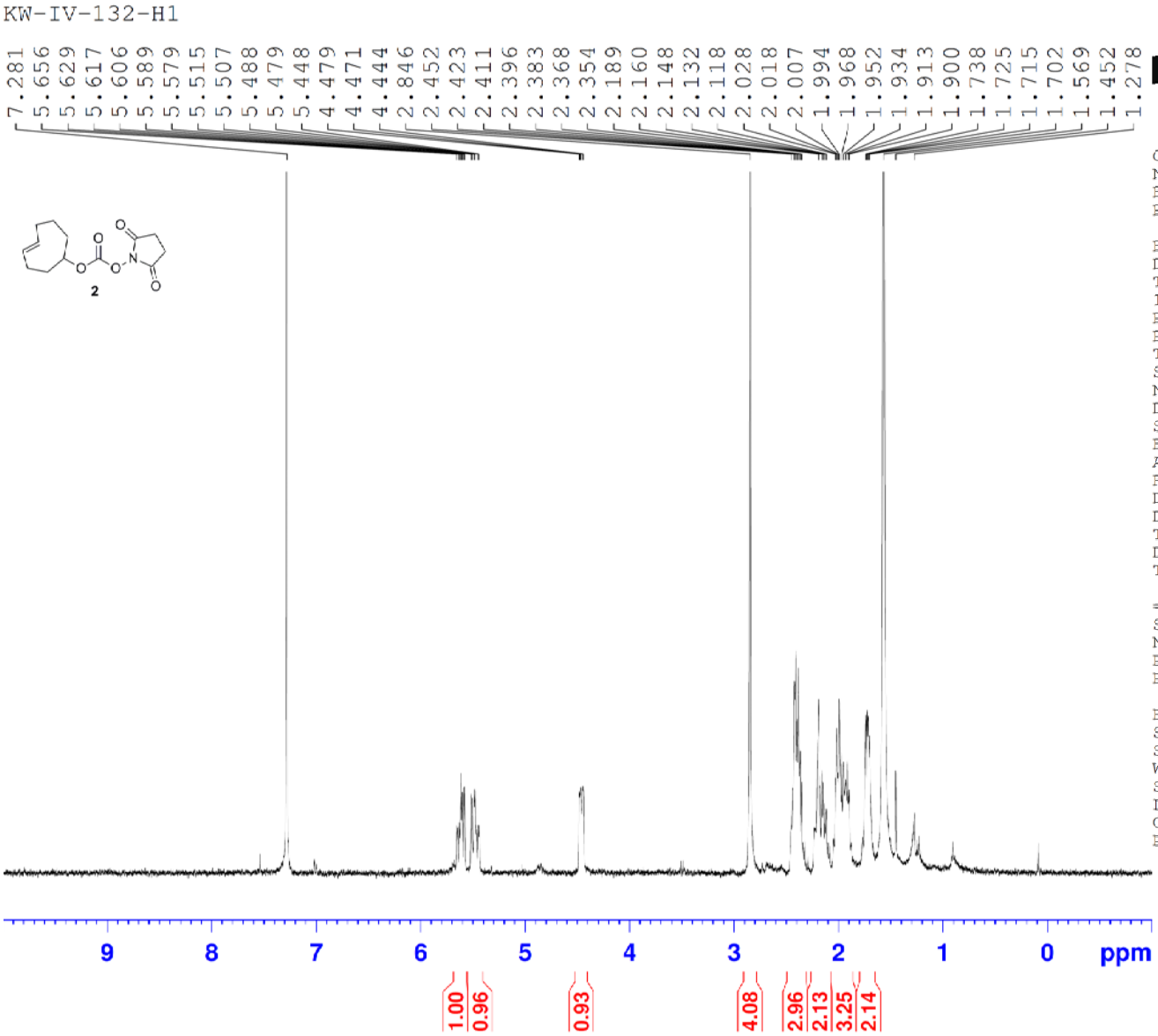
KW-IV-132-H1



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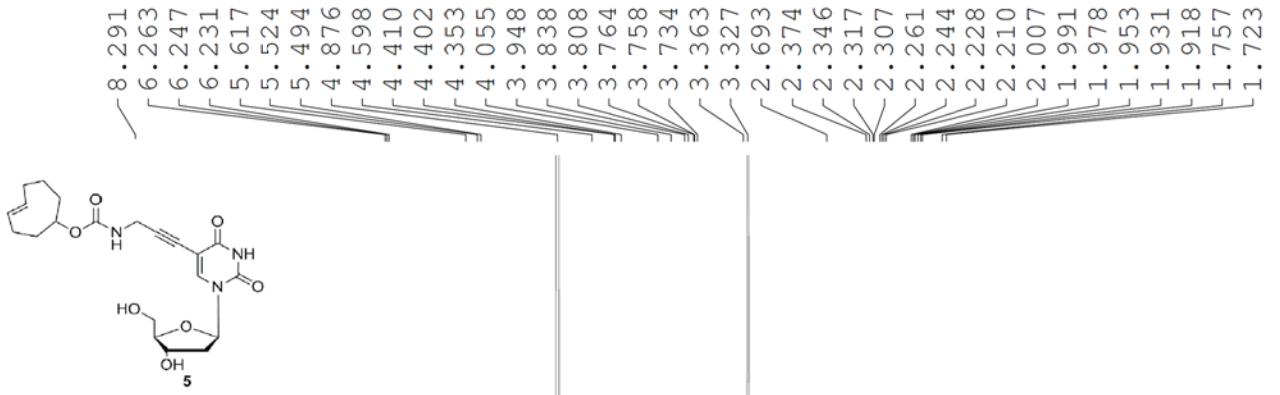

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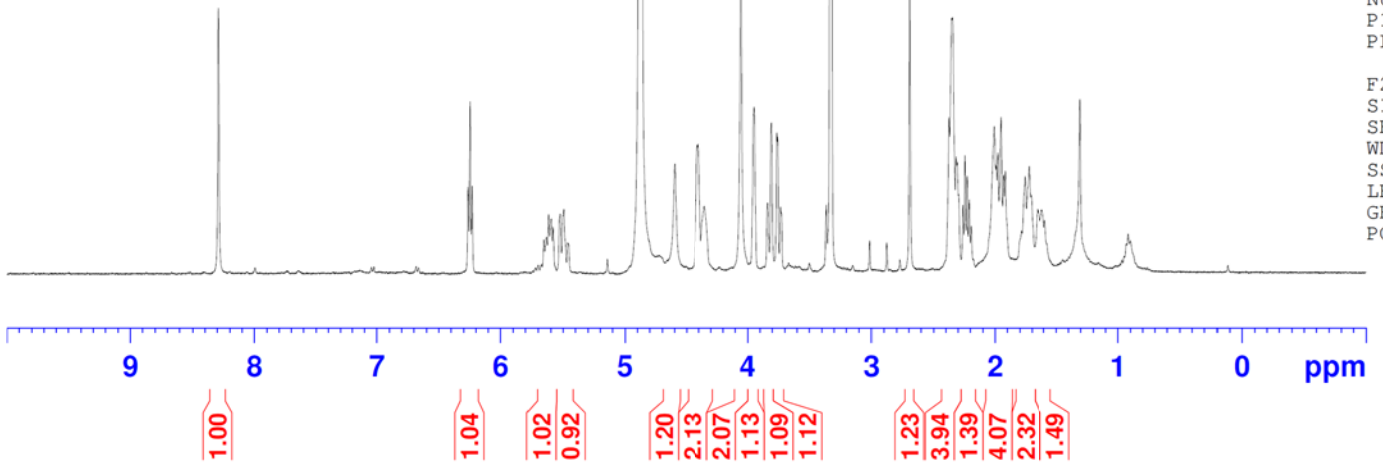

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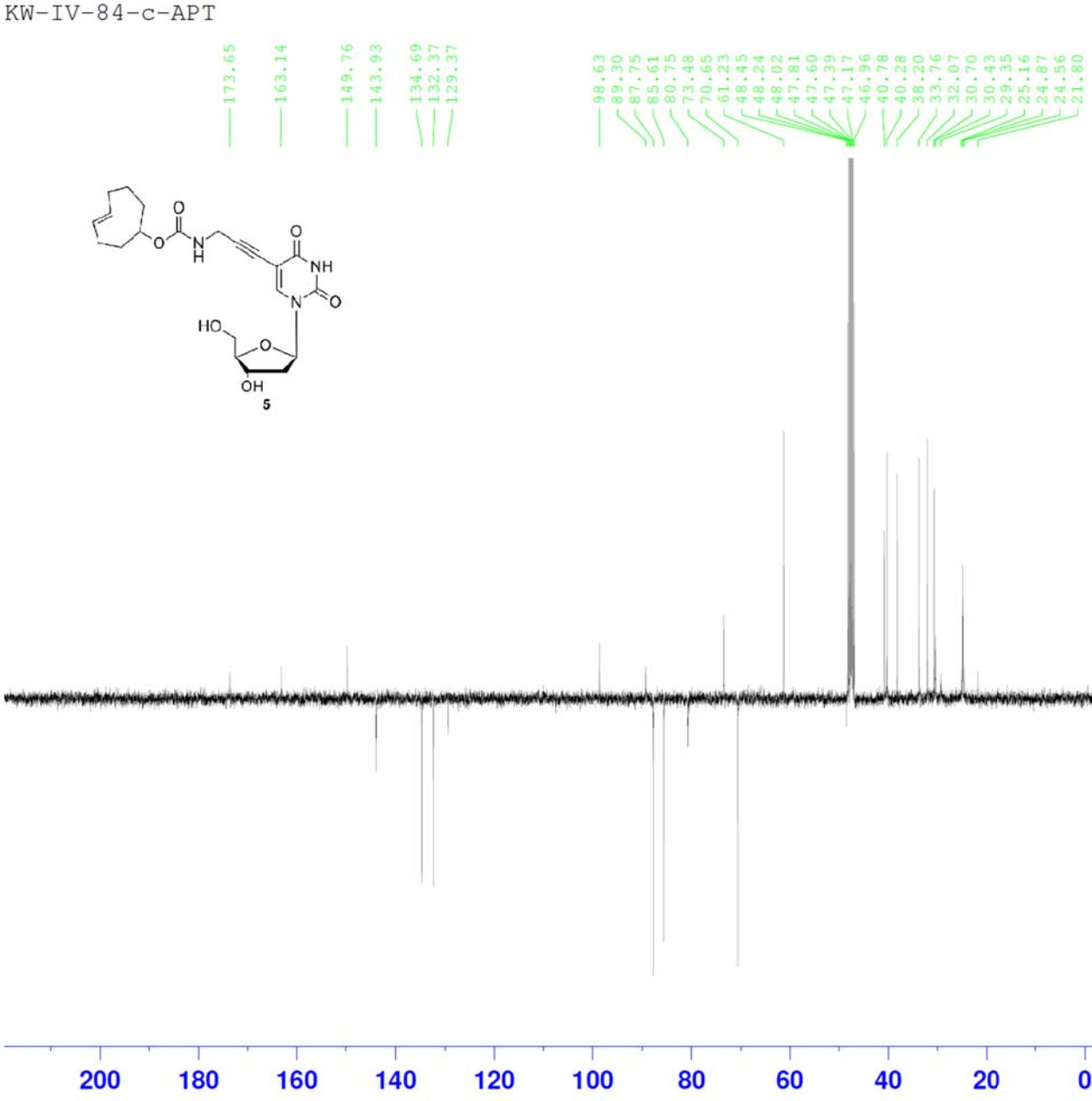

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DS                     4
SWH          24038.461 Hz
FIDRES        0.366798 Hz
AQ            1.3631488 sec
RG                   203
DW              20.800 usec
DE               6.50 usec
TE               298.0 K
CNST2        145.0000000
CNST11         1.0000000
D1           2.00000000 sec
D20          0.00689655 sec
TD0                    1

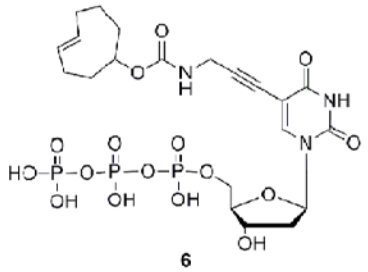
======== CHANNEL f1 ========
SFO1         100.6253446 MHz
NUC1                13C
P1                 9.00 usec
P2                18.00 usec
PLW1         62.00000000 W

======== CHANNEL f2 ========
SFO2         400.1416006 MHz
NUC2                1H
CPDPRG[2         waltz16
PCPD2             90.00 usec
PLW2         16.00000000 W
PLW12         0.36000001 W

F2 - Processing parameters
SI                 32768
SF           100.6152830 MHz
WDW                   EM
SSB       0
LB                 1.00 Hz
GB        0
PC                 1.40
```

KW-V-16-H1

8.056 6.208 6.191 6.175 5.661 5.647 5.624 5.606 5.519 5.491 5.454 4.926 4.845 4.828 4.767 4.761 4.698 4.584 4.567 4.562 4.264 4.237 4.132 4.018 4.002 2.326 2.315 2.292 2.274 2.257 1.983 1.929 1.892 1.868 1.844 1.832 1.629 1.621 1.591 1.571 1.454 1.201 1.186 0.011



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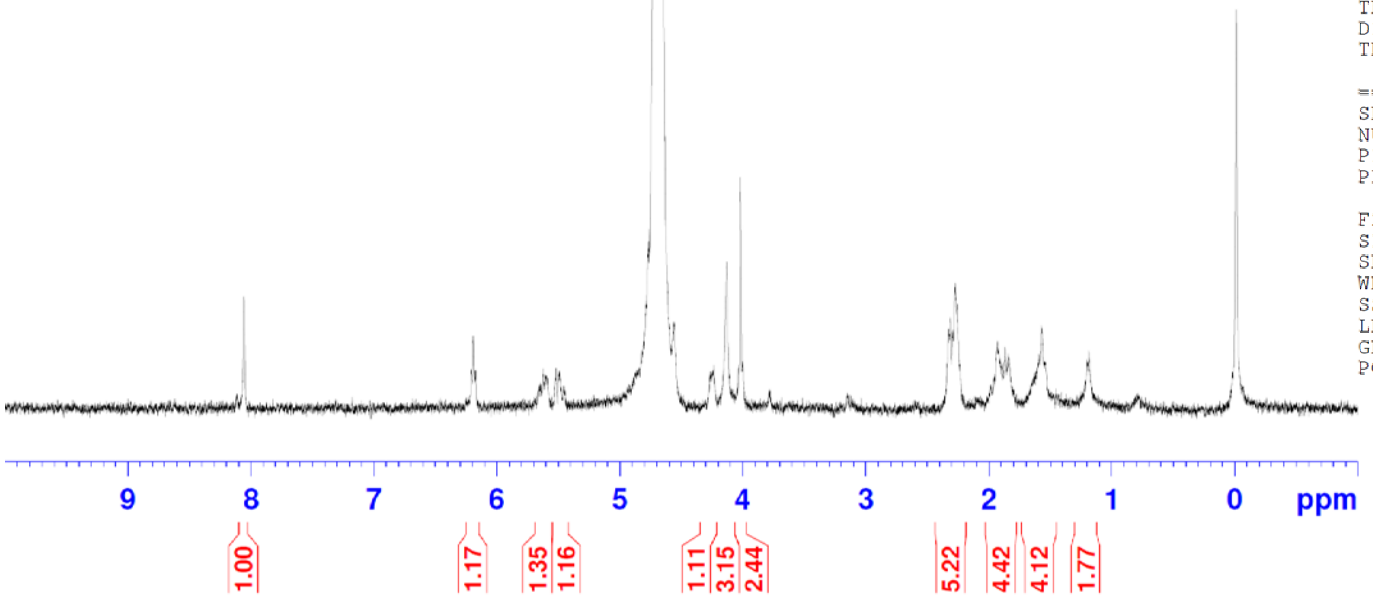


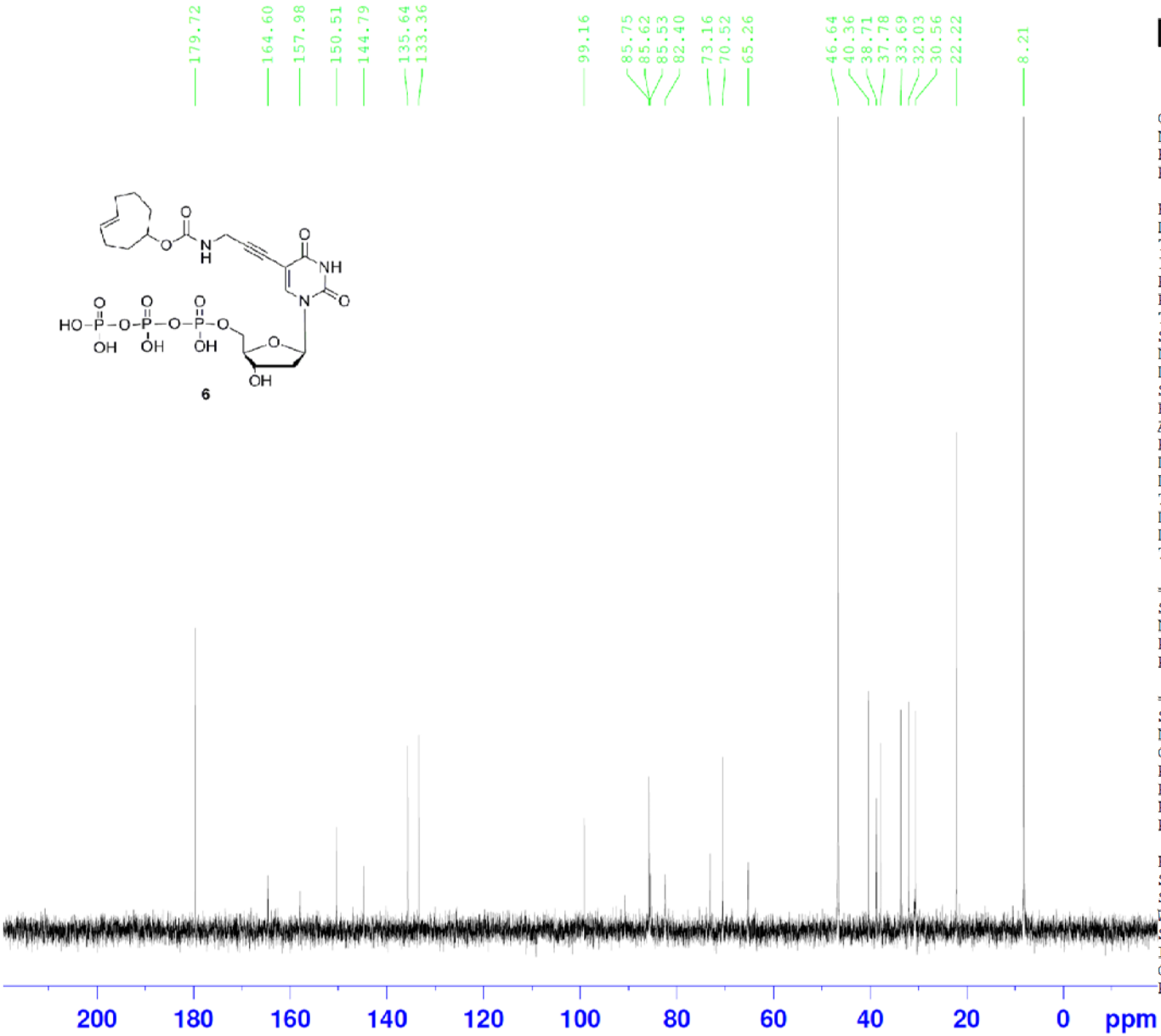
Current Data Parameters
NAME KW-V-16
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
Date_ 20131125
Time 13.10
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zg30
TD 65536
SOLVENT D2O
NS 16
DS 2
SWH 8012.820 Hz
FIDRES 0.122266 Hz
AQ 4.0894465 sec
RG 161
DW 62.400 usec
DE 6.50 usec
TE 298.1 K
D1 1.00000000 sec
TD0 1

======== CHANNEL f1 ========
SFO1 400.1424710 MHz
NUC1 1H
P1 13.50 usec
PLW1 16.00000000 W

F2 - Processing parameters
SI 65536
SF 400.1400000 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

KW-V-5-C13



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BRUKER

Current Data Parameters
NAME KW-V-5-b
EXPNO 2
PROCNO 1

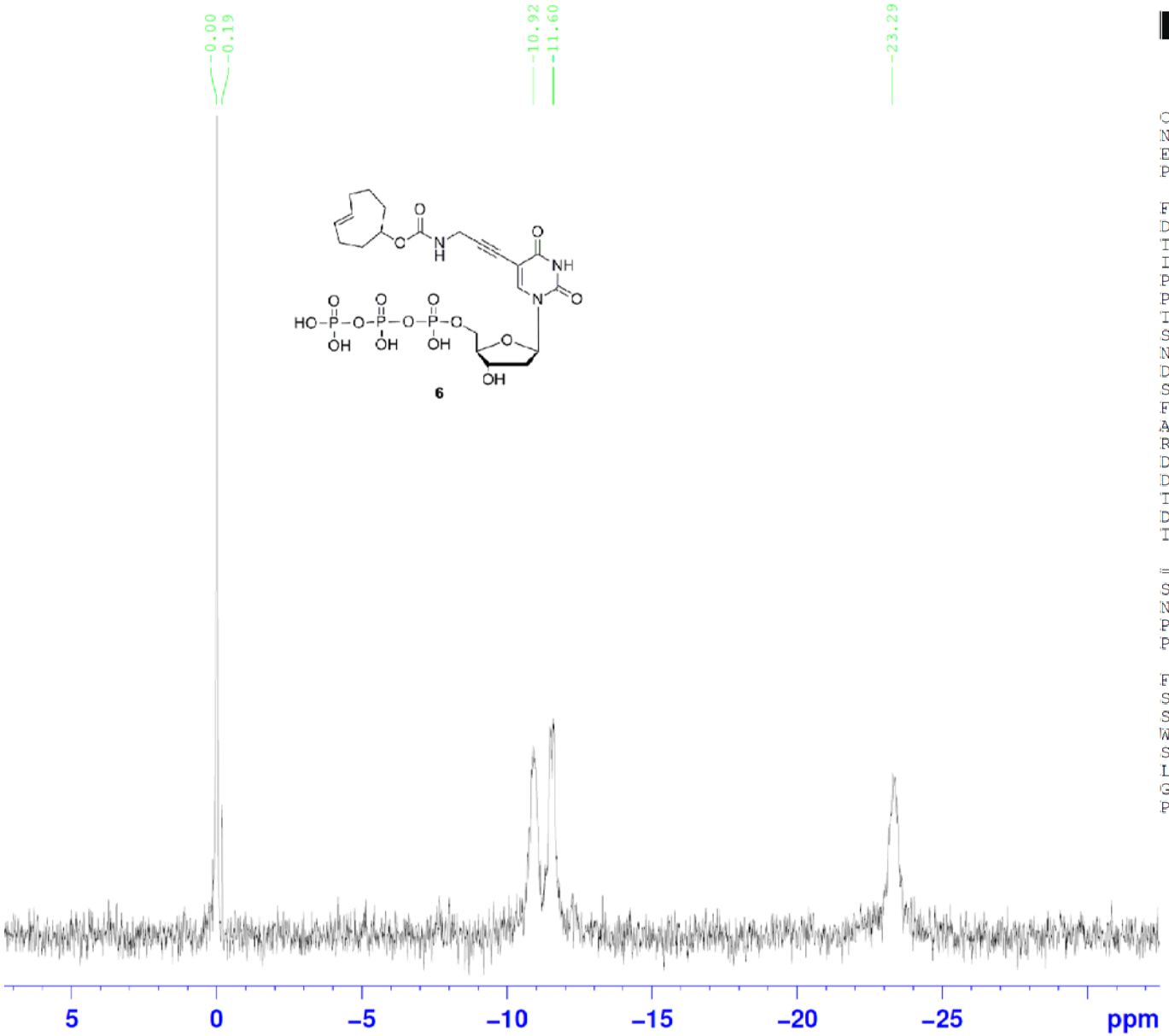
F2 - Acquisition Parameters
Date_ 20131119
Time 21.15
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zgpg30
TD 65536
SOLVENT D2O
NS 10240
DS 4
SWH 24038.461 Hz
FIDRES 0.366798 Hz
AQ 1.3631488 sec
RG 203
DW 20.800 usec
DE 6.50 usec
TE 298.4 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1

======== CHANNEL f1 ========
SFO1 100.6253441 MHz
NUC1 13C
P1 9.00 usec
PLW1 62.00000000 W

======== CHANNEL f2 ========
SFO2 400.1416006 MHz
NUC2 1H
CPDPRG[2 waltz16
PCPD2 90.00 usec
PLW2 16.00000000 W
PLW12 0.36000001 W
PLW13 0.29159999 W

F2 - Processing parameters
SI 32768
SF 100.6152830 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40

KW-V-5-P31-with dilute standard



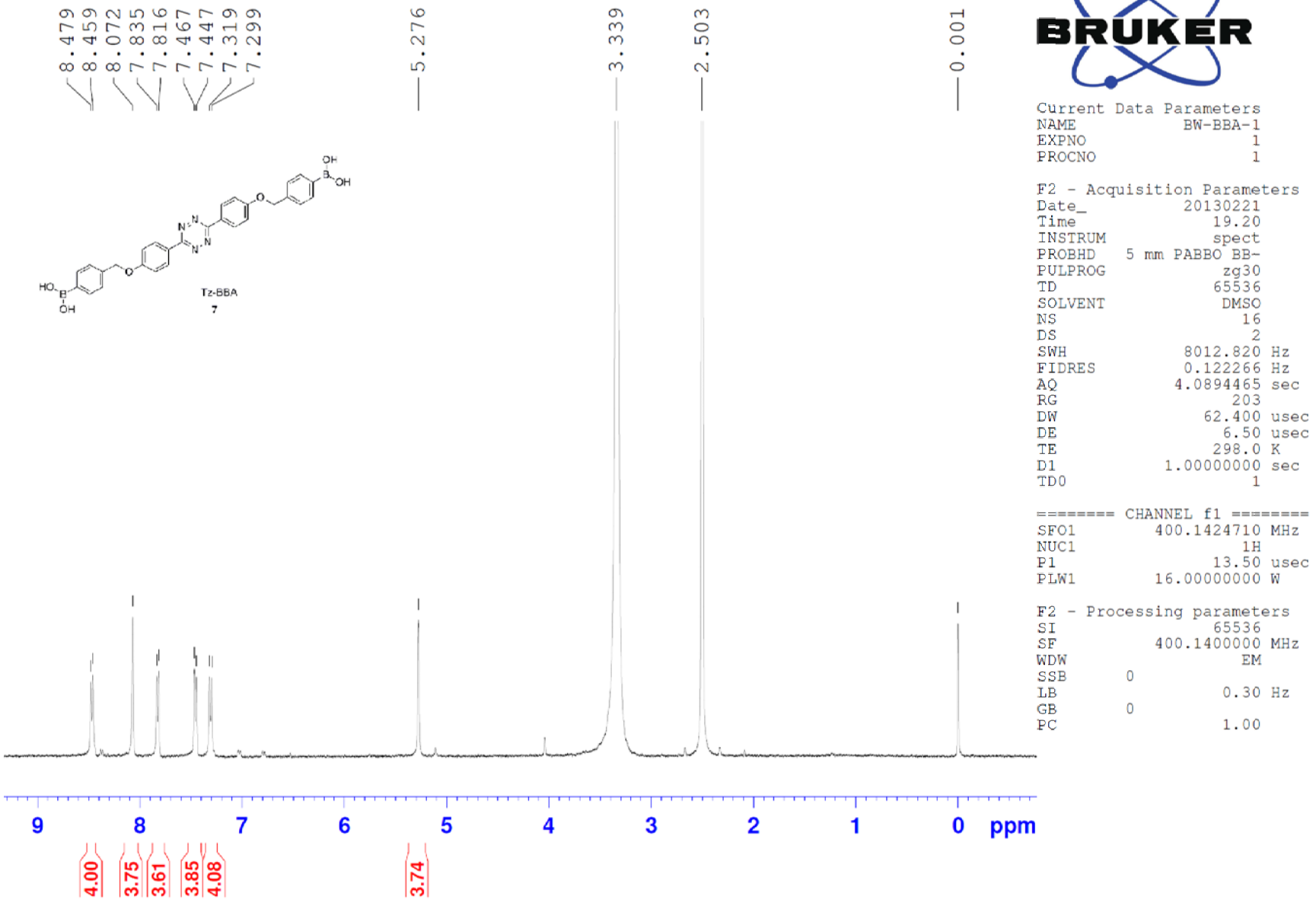

```
Current Data Parameters
NAME                  KW-V-5
EXPNO                      7
PROCNO                     1

F2 - Acquisition Parameters
Date_              20131104
Time                  13.56
INSTRUM               spect
PROBHD   5 mm PABBO BB-
PULPROG                zg30
TD                    65536
SOLVENT                D2O
NS                        64
DS                         4
SWH               64102.563 Hz
FIDRES             0.978127 Hz
AQ                0.5111808 sec
RG                      203
DW                  7.800 usec
DE                  6.50 usec
TE                  298.1 K
D1               2.00000000 sec
TD0                        1

======== CHANNEL f1 ========
SFO1            161.9715421 MHz
NUC1                    31P
P1                  14.00 usec
PLW1            12.50000000 W

F2 - Processing parameters
SI                     32768
SF              161.9796458 MHz
WDW                       EM
SSB          0
LB                  1.00 Hz
GB           0
PC                  1.40
```

BW-BBA-1-H1



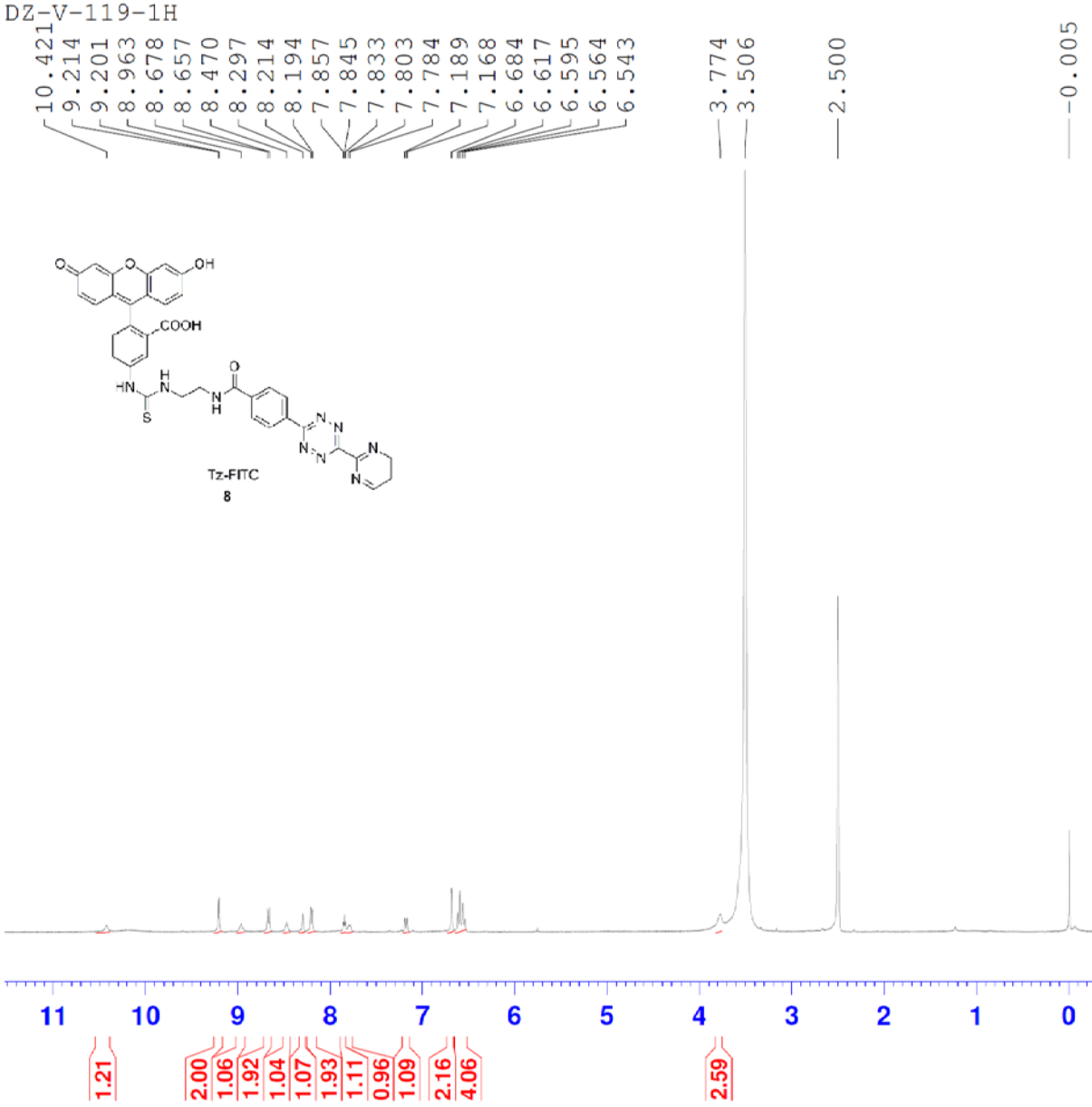

```
Current Data Parameters
NAME                  BW-BBA-1
EXPNO                        1
PROCNO                       1

F2 - Acquisition Parameters
Date_                 20130221
Time                     19.20
INSTRUM                  spect
PROBHD    5 mm PABBO BB-
PULPROG                  zg30
TD                      65536
SOLVENT                  DMSO
NS                         16
DS                          2
SWH                   8012.820 Hz
FIDRES                0.122266 Hz
AQ                 4.0894465 sec
RG                         203
DW                    62.400 usec
DE                     6.50 usec
TE                     298.0 K
D1                 1.00000000 sec
TD0                          1

======== CHANNEL f1 ========
SFO1               400.1424710 MHz
NUC1                        1H
P1                    13.50 usec
PLW1              16.00000000 W

F2 - Processing parameters
SI                      65536
SF                 400.1400000 MHz
WDW                        EM
SSB          0
LB                     0.30 Hz
GB           0
PC                     1.00
```

DZ-V-119-1H



BRUKER

```
Current Data Parameters
NAME                  DZ-V-119
EXPNO                        1
PROCNO                       1

F2 - Acquisition Parameters
Date_                 20130907
Time                     18.52
INSTRUM                  spect
PROBHD    5 mm PABBO BB-
PULPROG                  zg30
TD                      65536
SOLVENT                  DMSO
NS                         16
DS                          2
SWH                   8012.820 Hz
FIDRES                0.122266 Hz
AQ                    4.0894465 sec
RG                         203
DW                       62.400 usec
DE                        6.50 usec
TE                       296.3 K
D1                 1.00000000 sec
TD0                          1

======== CHANNEL f1 ========
SFO1               400.1424710 MHz
NUC1                        1H
P1                       13.50 usec
PLW1               16.00000000 W

F2 - Processing parameters
SI                          65536
SF                 400.1400013 MHz
WDW                         EM
SSB          0
LB                        0.30 Hz
GB           0
PC                        1.00
```