Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

SUPPORTING INFORMATION

33,6-Substituted-1,2,4,5-Tetrazines: Tuning Reaction Rates for Staged Labeling Applications

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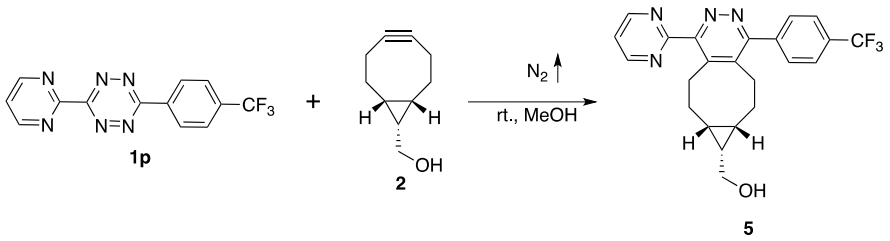
Materials and Methods

All reagents and solvents were reagent grade or were purified by standard methods before use. Column chromatography was carried out on flash silica gel (Sorbent 230–400 mesh). TLC analysis was conducted on silica gel plates (Sorbent Silica G UV254). NMR spectra were recorded at ^{1}H (400 MHz) and ^{13}C (100 MHz) on a Bruker instrument. Chemical shifts (δ values) and coupling constants (J values) are given in ppm and hertz, respectively, using solvents (^{1}H NMR, ^{13}C NMR) as the internal standard. Symmetric tetrazines **1a-1e**, BCN (**2**) were synthesized according to literature procedures. General synthesis procedure and characterization data of 3,6-substituted-1,2,4,5-tetrazines **1f-1p**, compound **6**, and compound **9** were already reported by our group.[20, 31]

Kinetics measurements of 3,6-substituted-1,2,4,5-tetrazines 1 with *exo*-BCN 2 or norbornene-2-carboxaldehyde 4. UV/Vis kinetic measurements: Separate solutions of pure tetrazines (**1**) and pure *exo*-BCN (**2**, >95-98 % by 1H-NMR) or norbornene-2-carboxaldehyde (**4**, 95%, sigma-aldrich), were prepared in HPLC-grade MeOH or CH3CN at room temperature. The stability of tetrazines (**1**) in MeOH or CH3CN was examined by monitoring their absorption at 295 nm- 330 nm. Solutions containing **1** (25 μM) and 10-18 fold excess of BCN (**2**) or tetrazines **1** (1 mM) and 10 fold excess of norbornene-2-carboxaldehyde (**4**) were pipetted into quartz cuvettes and thoroughly mixed. Curve fitting (Fitting equation: $Y=(Y_0 - Plateau)*exp(-K*X) + Plateau$) was conducted in Prism5 software.

1. Kinetics measurements of 1 with 2

1-1. Tetrazine **1p** with alkyne **2**



SI3

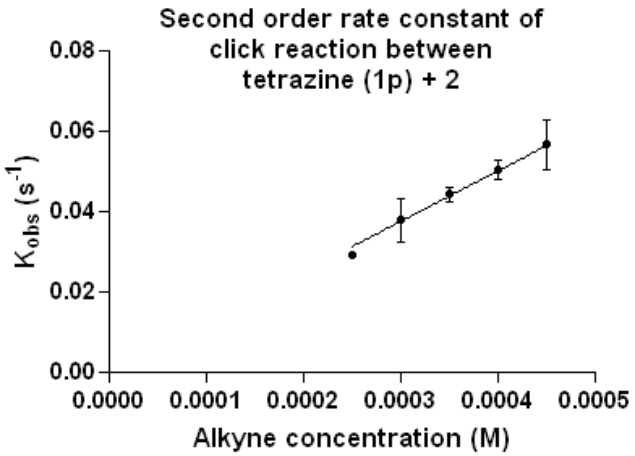
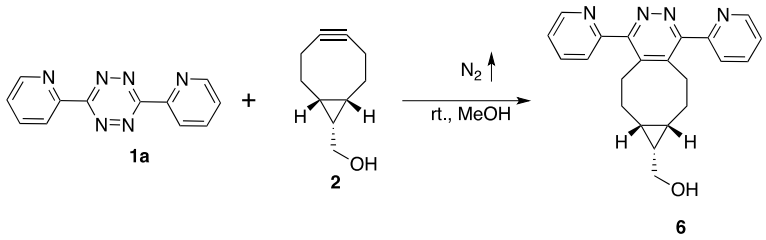


Figure S1. K_2 = 125 ±1 $M^{-1}s^{-1}$ (y = 125.5x, R^2 = 0.9901)
Concentration: Tetrazine (**1p)**: 25 μM,
exo-BCN (**2**): 250 μM, 300 μM, 350 μM, 400 μM, 450 μM.

1-2. Tetrazine **1a** with alkyne **2**



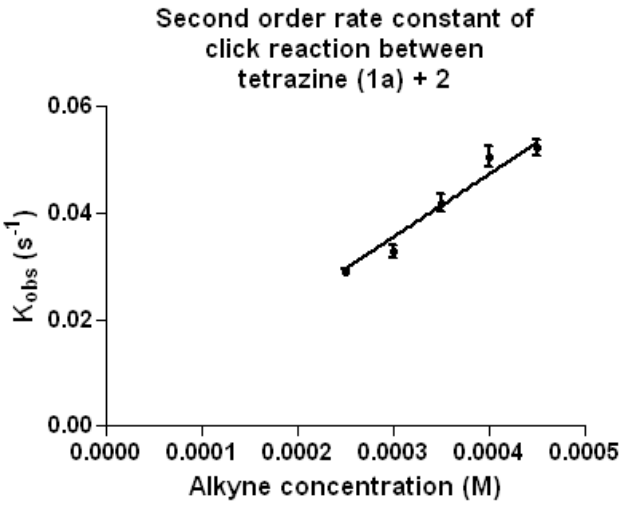
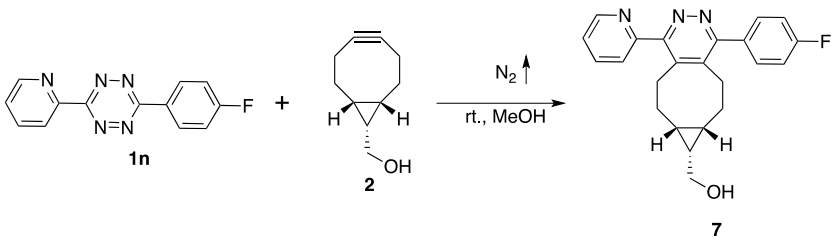


Figure S2. K_2 = 118 ±2 $M^{-1}s^{-1}$ **(**y = 118.33x, R^2= 0.9547**)**
Concentration: Tetrazine (**1a)**: 25 μM,
exo-BCN (**2**): 250 μM, 300 μM, 350 μM, 400 μM, 450 μM.

1-3. Tetrazine **1n** with alkyne **2**

SI4



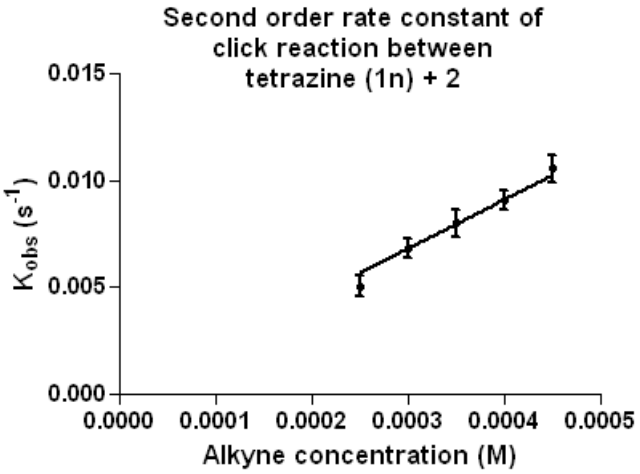


Figure S3. K_2 = 23 ± 0.5 $M^{-1}s^{-1}$ (y = 22.78x, R^2= 0.9705)
Concentration: Tetrazine (**1n)**: 25 μM,
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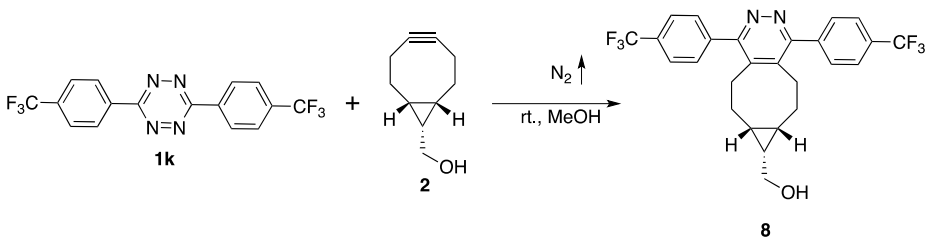
1-4. Tetrazine **1k** with alkyne **2**



SI5

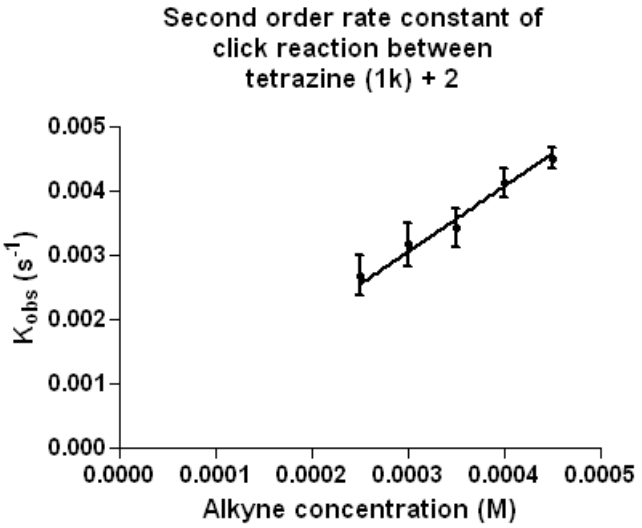


Figure S4. K_2 = 10 ±0.2 $M^{-1}s^{-1}$ (y = 10.206x, R^2= 0.9717)
Concentration: Tetrazine (**1k)**: 25 μM,
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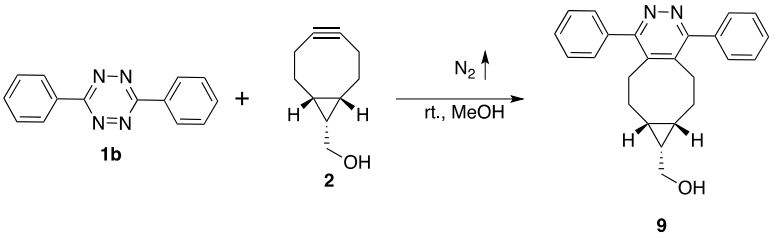
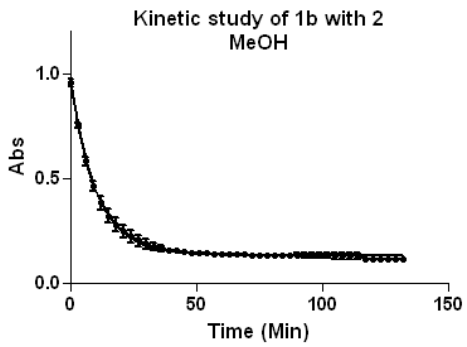
1-5. Tetrazine **1b** with alkyne **2**





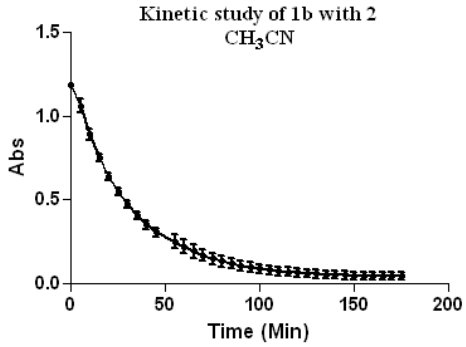


Figure S5. k_2 (MeOH) = 3.6 ±0.6 $M^{-1}s^{-1}$, k_1 = 0.0016 s^{-1} ($t_{1/2}$ = 7 min, R^2 = 0.9901)
k_2 (CH_3CN) = 1.2 ±0.1 $M^{-1}s^{-1}$, k_1 = 0.00054 s^{-1} ($t_{1/2}$ = 21 min, R^2 = 0.9993)
Concentration: Tetrazine (**1k)**: 25 μM, *exo*-BCN (**2**): 450 μM.

1-6. Tetrazine **1i** with alkyne **2**

SI6

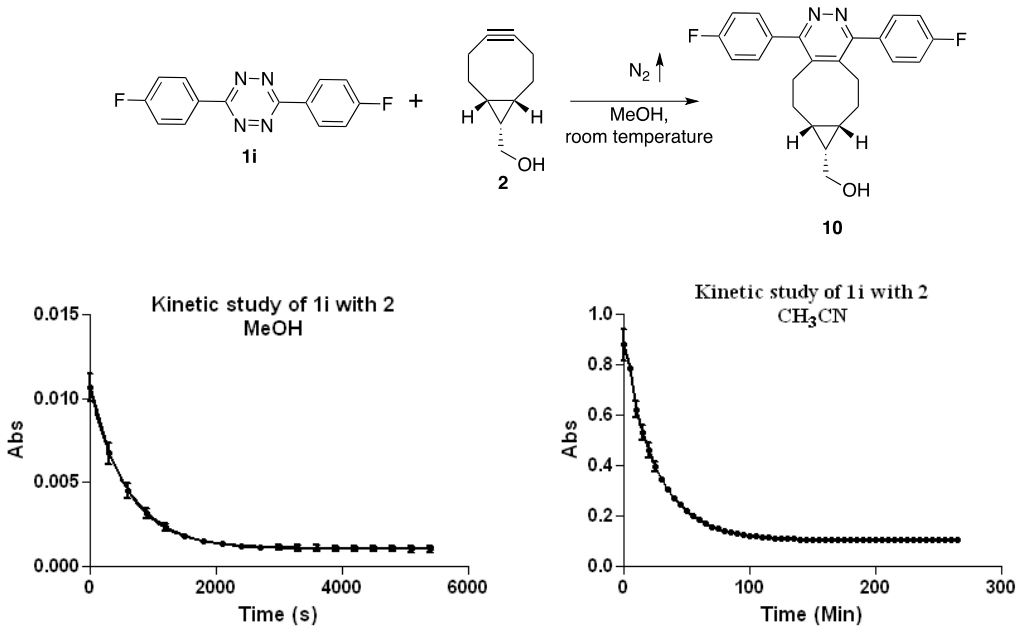


Figure S6. k_2 (MeOH) = 2.7 ±0.6 $M^{-1}s^{-1}$, k_1 = 0.0012s^{-1} ($t_{1/2}$ = 10 min, R^2 = 0.9999)
k_2 (CH_3CN) = 1.4 ±0.1 $M^{-1}s^{-1}$, k_1 = 0.00063 s^{-1} ($t_{1/2}$ = 18 min, R^2 = 0.9988)
Concentration: Tetrazine (**1i)**: 25 μM, *exo*-BCN (**2**): 450 μM.

1-7. Tetrazine **1d** with alkyne **2**

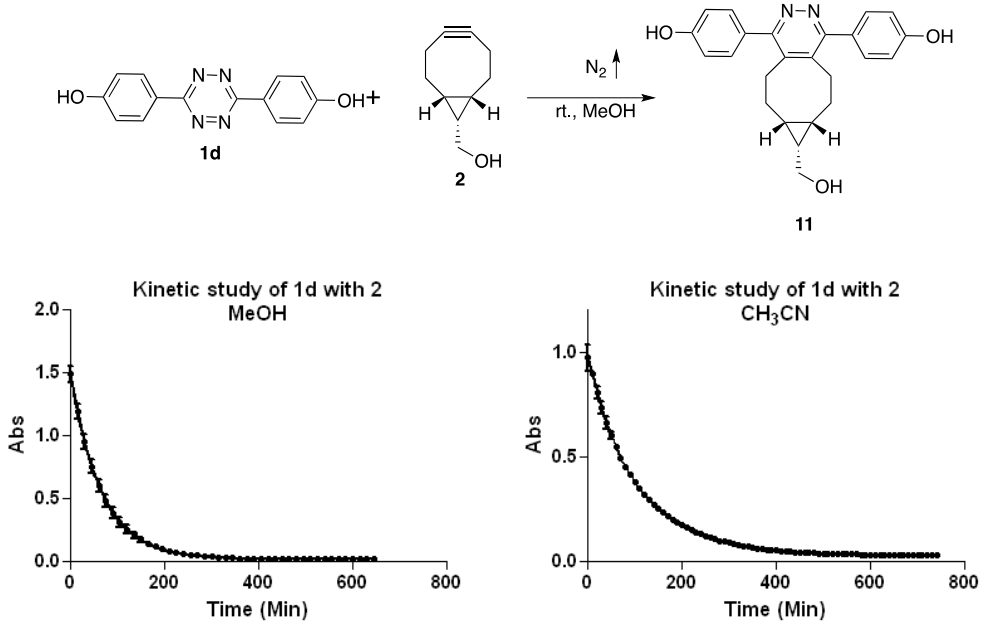


Figure S7. k_2 (MeOH) = 0.58 ±0.02 $M^{-1}s^{-1}$, k_1 = 0.00026s^{-1} ($t_{1/2}$ = 45 min, R^2 = 0.9944)
k_2 (CH_3CN) = 0.36 ±0.01 $M^{-1}s^{-1}$, k_1 = 0.00016 s^{-1} ($t_{1/2}$ = 72 min, R^2 = 0.9995)
Concentration: Tetrazine (**1d)**: 25 μM, *exo*-BCN (**2**): 450 μM.

1-8. Tetrazine **1e** with alkyne **2**

SI7

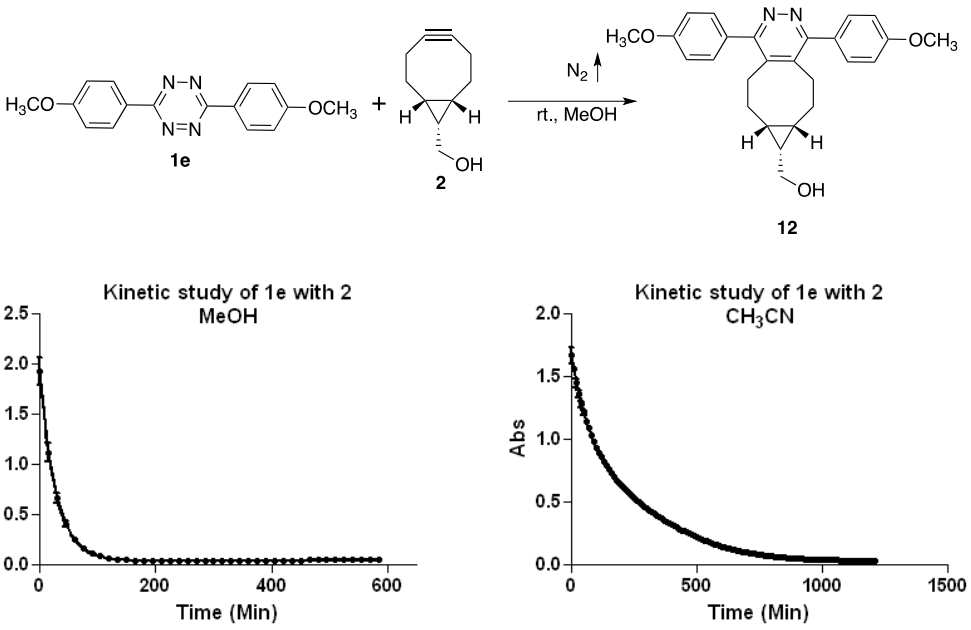


Figure S8. k_2 (MeOH) = 1.4 ±0.03 $M^{-1}s^{-1}$, k_1 = 0.00061s^{-1} ($t_{1/2}$ = 19 min, R^2 = 0.9926)
k_2 (CH_3CN) = 0.16 ±0.01 $M^{-1}s^{-1}$, k_1 = 0.000073 s^{-1} ($t_{1/2}$ = 158 min, R^2 = 0.9949)
Concentration: Tetrazine (**1e)**: 25 μM, *exo*-BCN (**2**): 450 μM.

1-9. Tetrazine **1c** with alkyne **2**

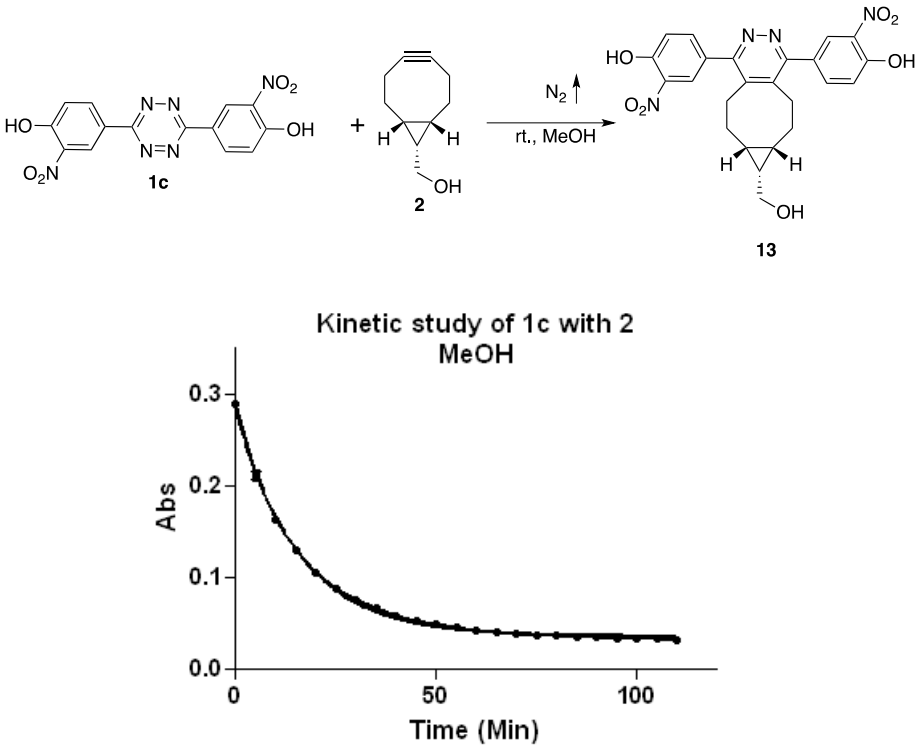
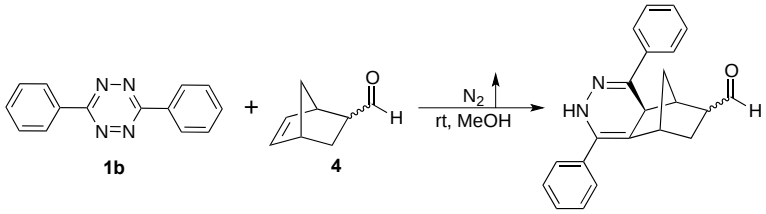


Figure S9. k_2 = 2.3 ±0.03 $M^{-1}s^{-1}$, k_1 = 0.0011s^{-1} ($t_{1/2}$ = 11 min, R^2 = 0.9975)
Concentration: Tetrazine (**1e)**: 25 μM, *exo*-BCN (**2**): 450 μM.

2. Kinetics measurements of 1 with 4

SI8

2-1. Tetrazine **1b** with alkyne **4**



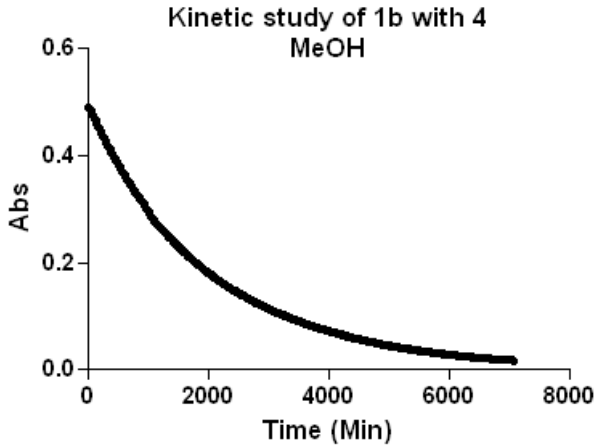
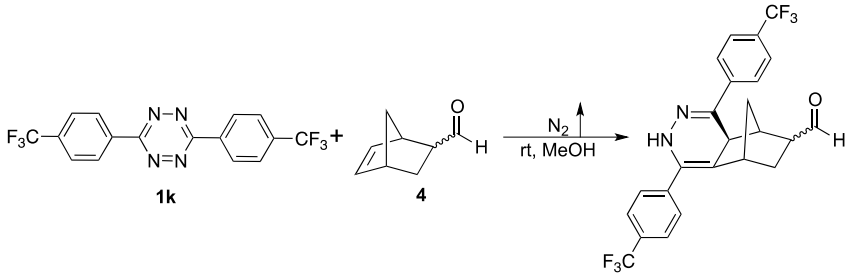
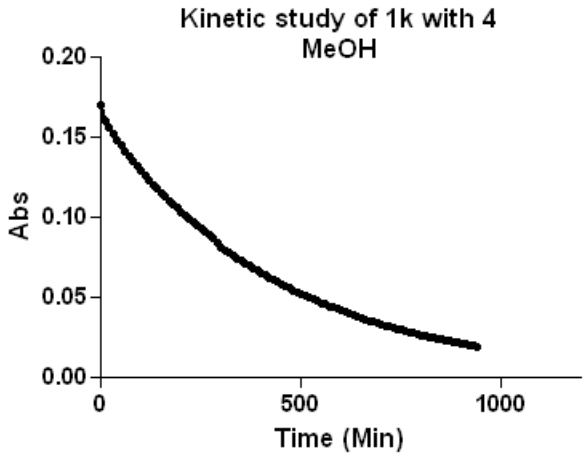


Figure S2-1. k_2 = 0.0085 $M^{-1}s^{-1}$, k_1 = 8.5 x $10^{-6}s^{-1}$ ($t_{1/2}$ = 1364 min, R^2 = 0.9998)
Concentration: Tetrazine (**1b)**: 1 mM, *exo*-BCN (**2**): 10 mM.

2-2. Tetrazine **1k** with alkyne **4**

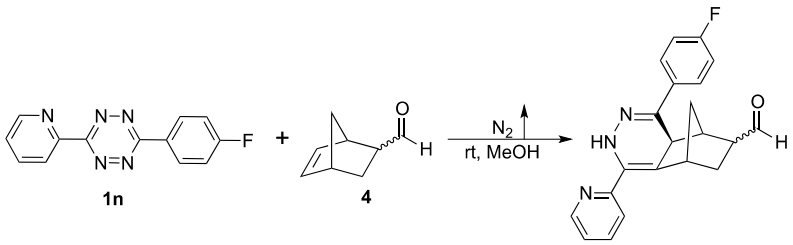




SI9

Figure S2-2. k_2 = 0.039 $M^{-1}s^{-1}$, k_1 = 3.9 x $10^{-5}s^{-1}$ ($t_{1/2}$ = 297 min, R^2 = 0.9996)
Concentration: Tetrazine (**1k)**: 1 mM, *exo*-BCN (**2**): 10 mM.

2-3. Tetrazine **1n** with alkyne **4**



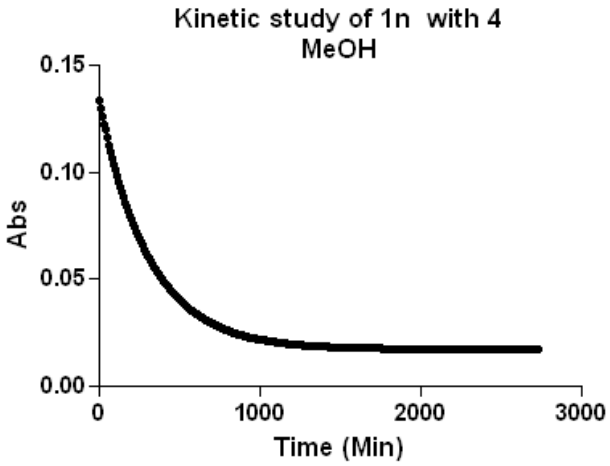
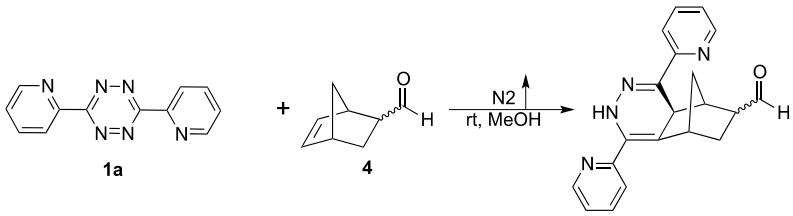
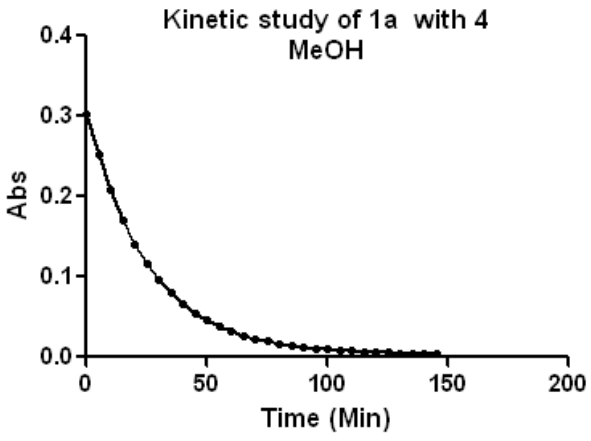


Figure S2-3. k_2 = 0.054 $M^{-1}s^{-1}$, k_1 = 5.4 x $10^{-5}s^{-1}$ ($t_{1/2}$ = 214 min, R^2 = 1.000)
Concentration: Tetrazine (**1k)**: 1 mM, *exo*-BCN (**2**): 10 mM.

2-4. Tetrazine **1a** with alkyne **4**

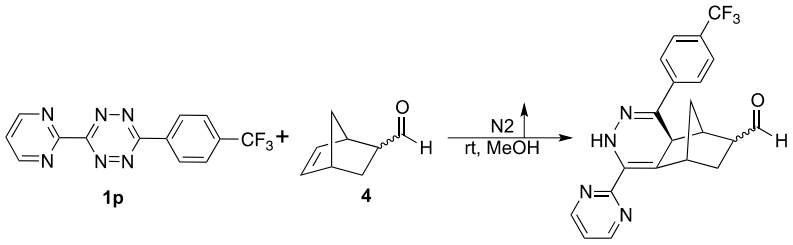




SI10

Figure S2-4. k_2 = 0.65 $M^{-1}s^{-1}$, k_1 = 6.5 x $10^{-4}s^{-1}$ ($t_{1/2}$ = 18 min, R^2 = 1.000)
Concentration: Tetrazine (**1k)**: 1 mM, *exo*-BCN (**2**): 10 mM.

2-5. Tetrazine **1p** with alkyne **4**



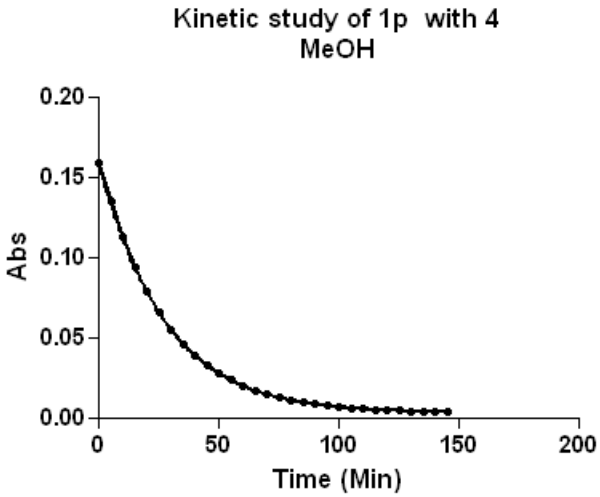
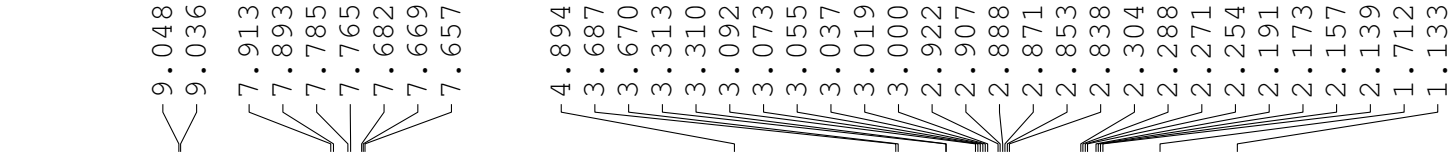
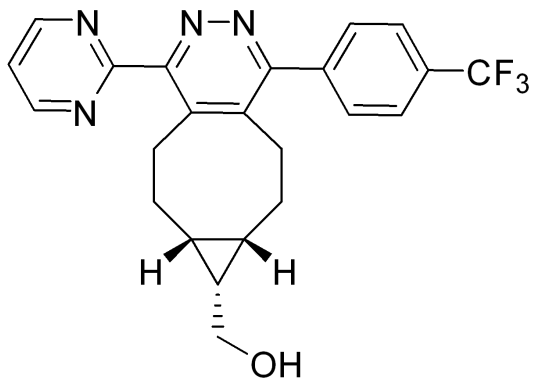


Figure S2-5. k_2 = 0.61 $M^{-1}s^{-1}$, k_1 = 6.1 x $10^{-4}s^{-1}$ ($t_{1/2}$ = 19 min, R^2 = 0.9999)
Concentration: Tetrazine (**1k)**: 1 mM, *exo*-BCN (**2**): 10 mM.

SI11

DZ-V-53-C-1H





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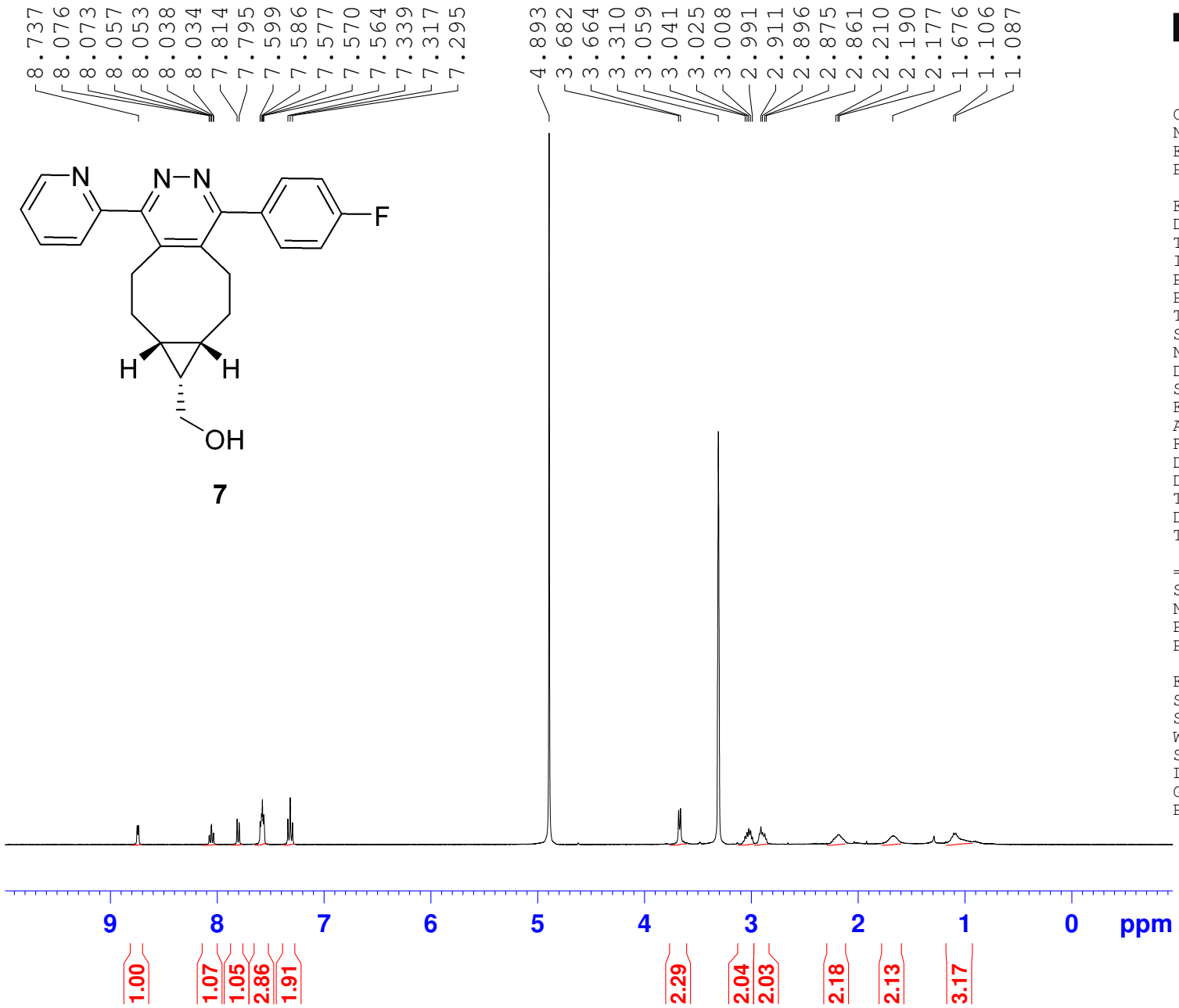

BRUKER

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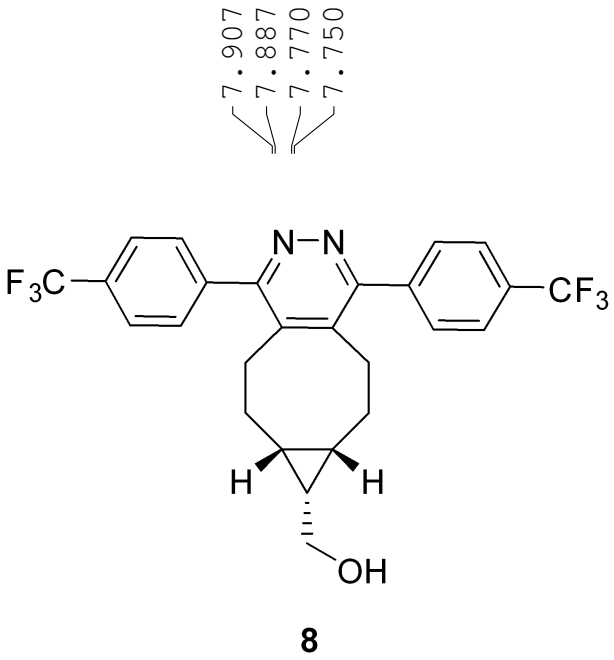

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ZYQ-2-41-1-1H

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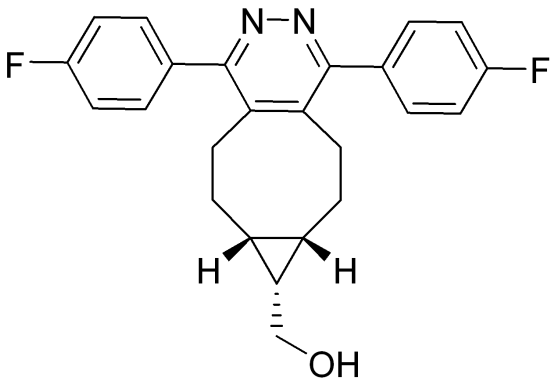

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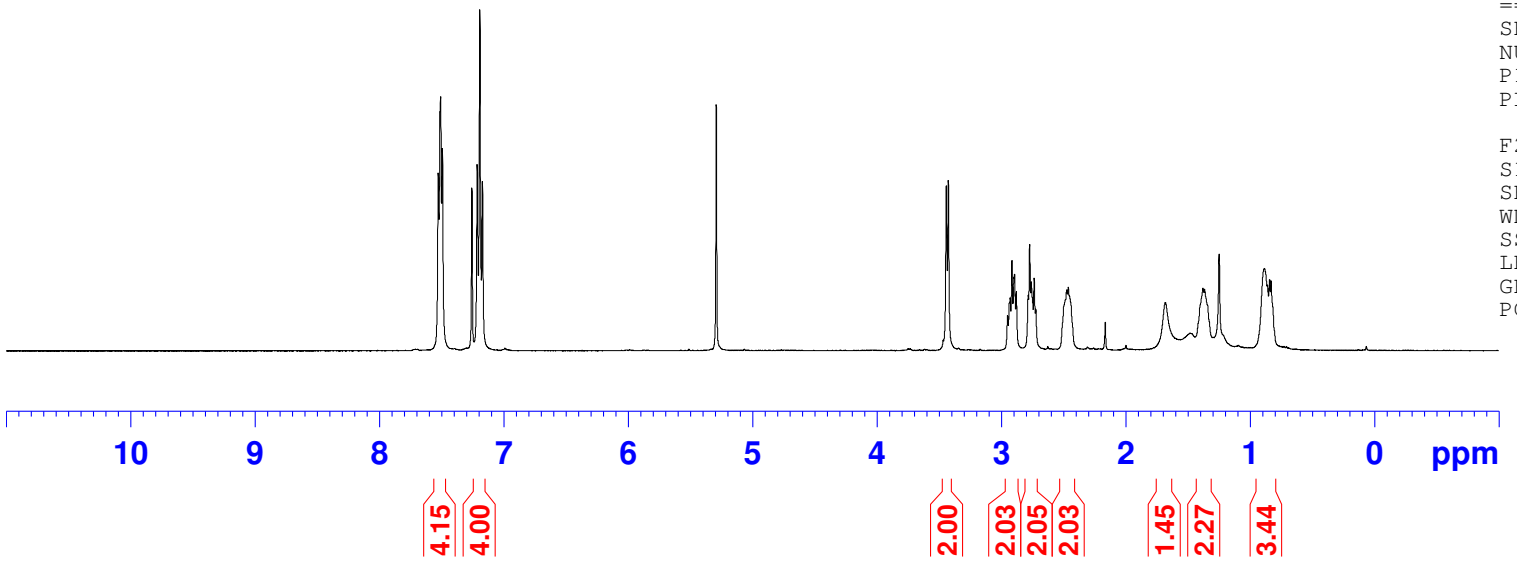

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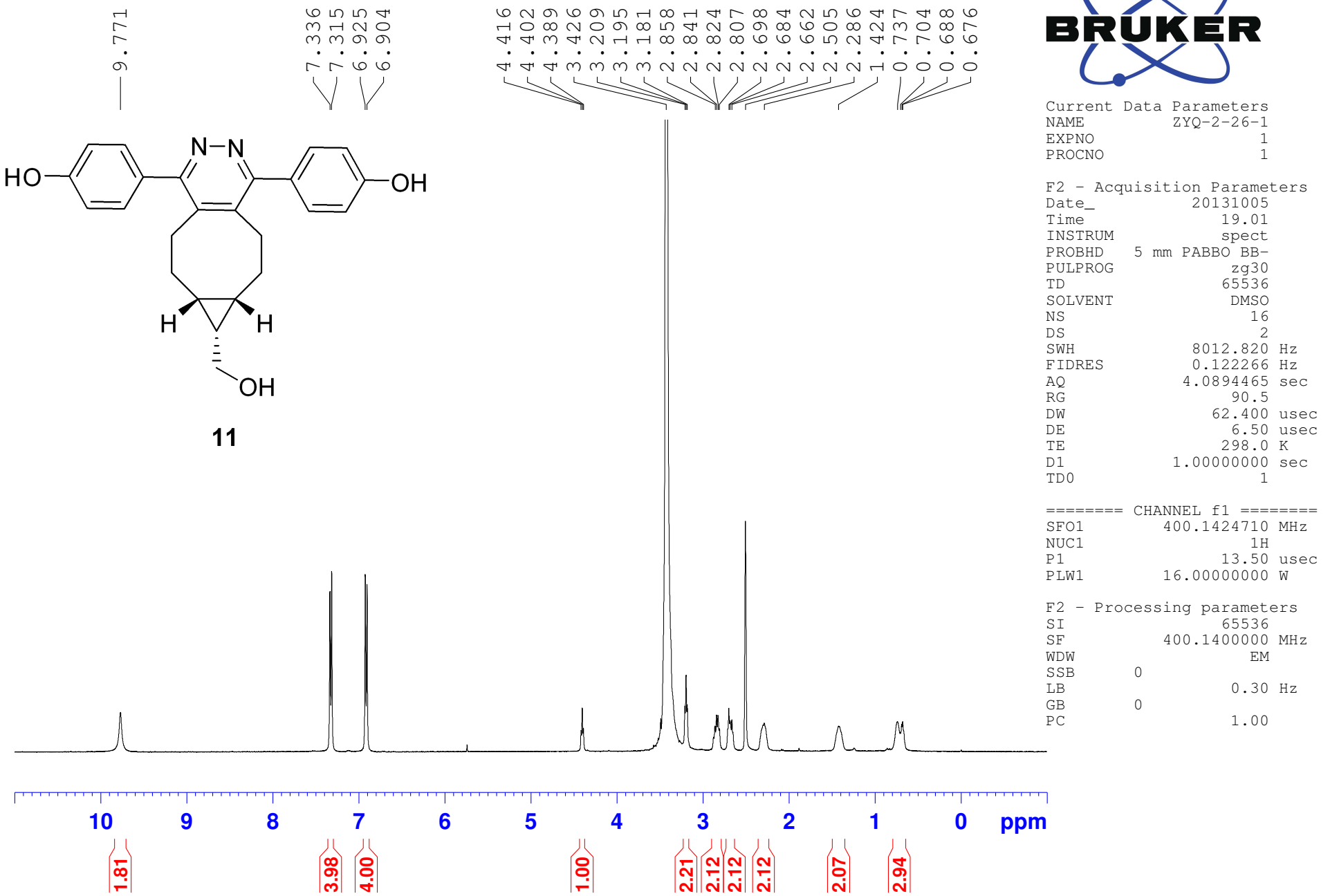


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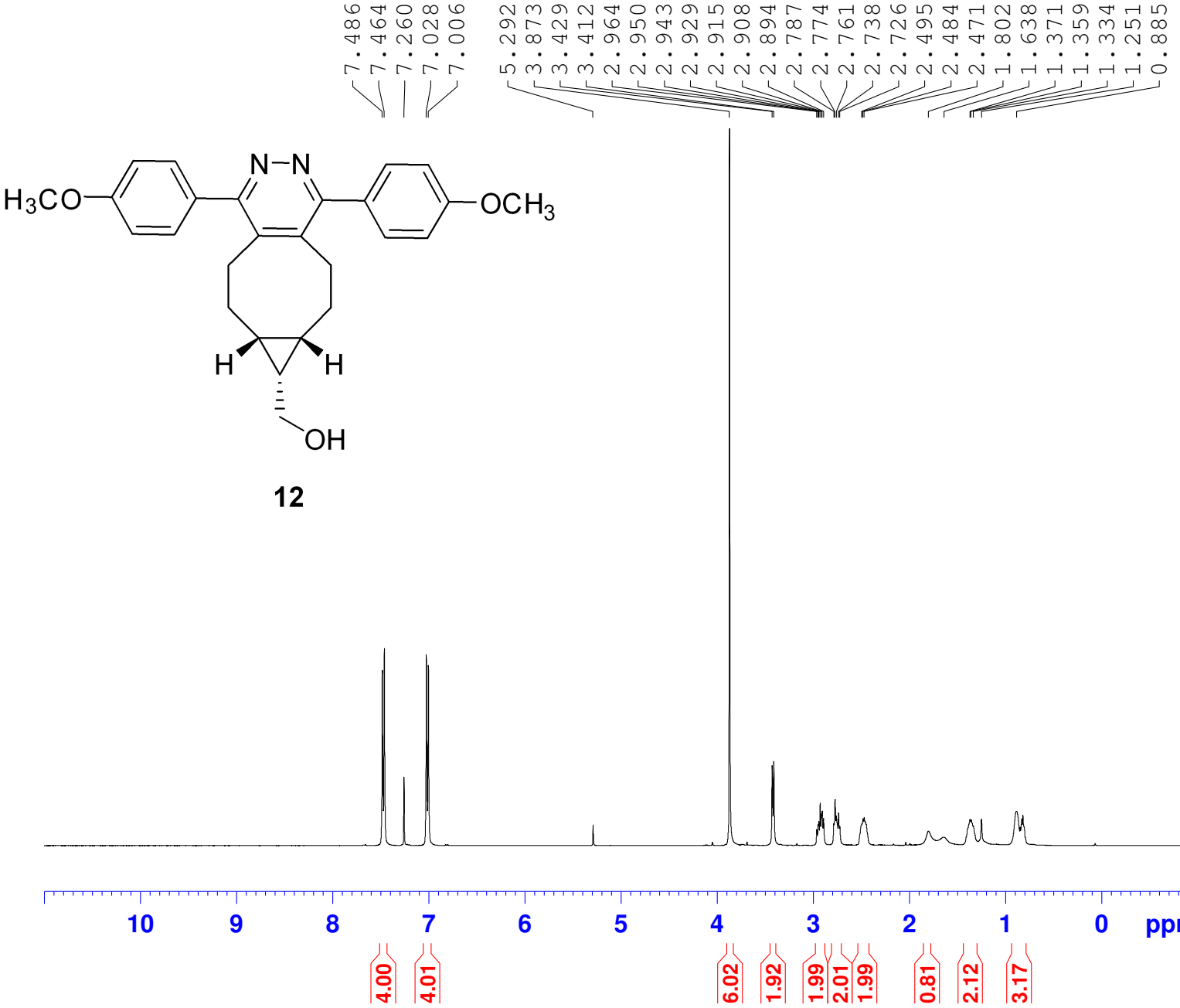
PC                 1.00
```

ZYQ-2-42-1-1H



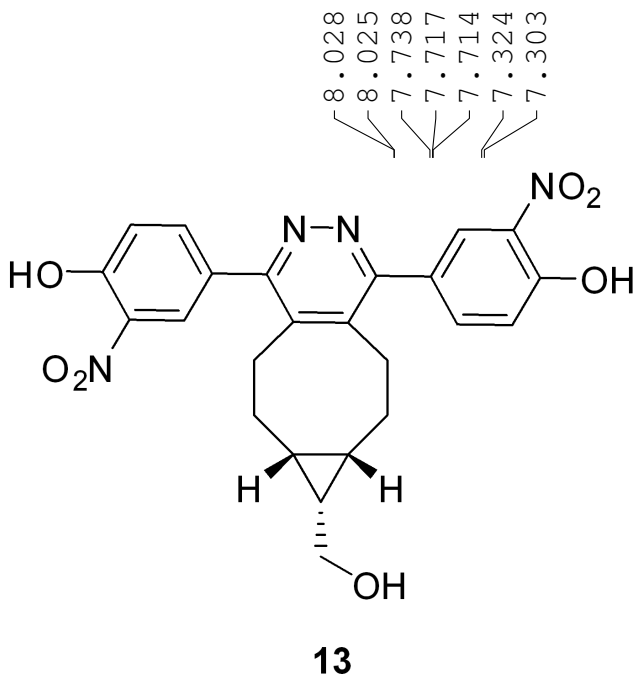

```
Current Data Parameters
NAME          ZYQ-2-42-1
EXPNO                  1
PROCNO                 1

F2 - Acquisition Parameters
Date_           20131015
Time               18.42
INSTRUM            spect
PROBHD   5 mm PABBO BB-
PULPROG             zg30
TD                 65536
SOLVENT            CDCl3
NS                    16
DS                     2
SWH             8012.820 Hz
FIDRES          0.122266 Hz
AQ              4.0894465 sec
RG                   128
DW                62.400 usec
DE                 6.50 usec
TE                297.0 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           400.1400086 MHz
WDW                   EM
SSB       0
LB                 0.30 Hz
GB        0
PC                 1.00
```

ZYQ-2-25-1-1H



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BRUKER

```
Current Data Parameters
NAME          ZYQ-2-25-1
EXPNO                  3
PROCNO                 1

F2 - Acquisition Parameters
Date_           20131005
Time               18.55
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                  90.5
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
```