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Supporting Information

An Extended Approach for the Development of Fluorogenic *trans*-Cyclooctene–Tetrazine Cycloadditions

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Content

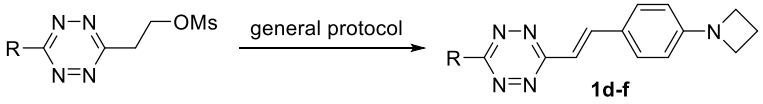
General information

The chemicals were obtained from commercial suppliers and were used without further purification. Reactions with air- and moisture-sensitive reactants were performed in anhydrous solvents under nitrogen or argon atmosphere. Photochemical reactions (synthesis of TCOs) were performed in a RPR-200 Rayonet reaction chamber equipped with 16 Hg-quartz iodine lamps (2537 Å) from *Southern New England Ultraviolet Company*. The continuous flow system during the photoreaction was produced by a STEPDOS 03 RC membrane-metering pump from *KNF*. Column chromatography was carried out on silica gel 60A (particle size: 40-60 µm) from *Acros Organics*. Mixtures of solvents are each stated as volume fractions. For flash column chromatography a Combi*Flash*® Rf+ from *Teledyne ISCO* was used. Thin-layer chromatography was performed on aluminum sheets from *Merck* (silica gel 60 F254, 20 × 20 cm). Chromatograms were visualized by UV light (λ = 254 nm/ 366 nm) or by staining with $KMnO_4$ solution. ^{1}H- and ^{13}C-NMR spectra were measured on a Bruker Avance III™ HD 400 MHz NMR system equipped with Prodigy cryo-probe. Chemical shifts δ are quoted in ppm in relation to the chemical shift of the residual non-deuterated solvent peak ($CDCl_3$: δ(^{1}H) = 7.26, δ(^{13}C) = 77.16). High-resolution mass spectra were recorded on an *Agilent* 5975C MSD Quadrupol, Q-Tof micro from *Waters* or LTQ Orbitrap XL from *Thermo Fisher Scientific*. HPLC-MS measurements were performed on an LCMS-2020 system from *Shimadzu* equipped either with a Luna® C18(2) column (3µm, 100A, 100 × 4.6 mm) or a Kinetex® C18 column (2.6µm, 100A, 100 × 4.6 mm). UV/VIS spectroscopy was performed on a Cary 60 UV/Vis spectrophotometer from *Agilent Technologies*. Data from kinetic experiments were processed using OriginPro 9.1 software. Fluorescence measurements were performed on a FluoroMax 4 spectrofluorometer (Jobin Yvon, Horiba) from *Perkin Elmer* equipped with a 450 W xenon lamp and a single cuvette reader using the dye nile red in MeOH as standard for determination of fluorescence quantum yields.

Synthetic procedures

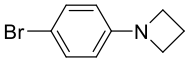
The synthesis of tetrazines **1a-c** has been published in one of our earlier publications.[1]

General procedure for the synthesis of 1d-f



To a Schlenk vessel we added mesilated starting tetrazine[1] (50 mg), P(*o*-Tol)$_3$ (40 mol%), $Pd_2(dba)_3$ (10 mol%) and 1-(4-bromophenyl)azetidine (1.5 equiv). The vessel was purged with argon and 5 mL of degassed anhydrous 1,4-dioxane was added via syringe followed by Cy_2NMe (3 equiv). The reaction mixture was vigorously stirred at 70 °C and cooled to RT after 20 h. Water (10 mL) was added and the mixture was extracted with DCM (3 × 5 mL), the combined extracts were dried over Na_2SO_4, filtered and evaporated.

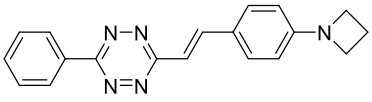
1-(4-Bromophenyl)azetidine



To a dry flask under an argon atmosphere was added azetidine hydrochloride (100 mg, 1.07 mmol), 1-bromo-4-iodobenzene (454 mg, 1.60 mmol, 1.5 equiv), Xantphos (62 mg, 0.107 mmol, 10 mol%), $Pd_2(dba)_3$ (50 mg, 0.053 mmol, 5 mol%) and *t*BuONa (308 mg, 3.204 mmol, 3 equiv). Then, anhydrous degassed 1,4-dioxane was added (10 mL) and the mixture was stirred at 95 °C for 12 h. Then, it was cooled to RT, brine (10 mL) and water (20 mL) were added and the mixture was extracted with Et_2O (2 × 50 mL). Collected extracts were dried over Na_2SO_4, filtered and evaporated.

PURIFICATION: flash chromatography, gradient PE → PE/EA 9:1
YIELD: 70 mg (31%)
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.25 – 7.30 (m, 2H), 6.29 – 6.33 (m, 2H), 3.85 (t, *J* = 7.2, 4H), 2.36 (pent, *J* = 7.2, 2H).
^{13}C NMR (101 MHz, $CDCl_3$): δ 151.2, 131.7, 113.1, 109.3, 52.6, 17.0.
HRMS (EI+) calcd. for $C_9H_{10}N_{Br}^+$ $[M]^+$: 210.9997, found 210.9995

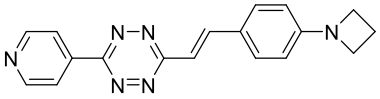
(*E*)-3-(4-Azetidin-1-yl)styryl-6-phenyl-1,2,4,5-tetrazine **1d**



PURIFICATION: column chromatography, PE/DCM 1:4
YIELD: 32 mg (57%)
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.56 – 8.61 (m, 2H), 8.29 (d, *J* = 16.1, 1H), 7.54 – 7.61 (m, 5H), 7.26 (d, *J* = 16.1, 1H), 6.42 – 6.47 (m, 2H), 3.99 (t, *J* = 7.3, 4H), 2.42 (pent, *J* = 7.3, 2H).

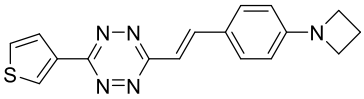
^{13}C NMR (101 MHz, $CDCl_3$): δ 165.4, 162.6, 153.1, 142.0, 132.4, 132.2, 129.9, 129.3, 127.7, 124.2, 115.4, 111.1, 52.0, 16.8.
HRMS (APCI+) calcd. for $C_{19}H_{18}N_5^+$ $[M+H]^+$: 316.1557, found 316.1557.

(*E*)-3-(4-Azetidin-1-yl)styryl)-6-(pyridin-4-yl)-1,2,4,5-tetrazine **1e**



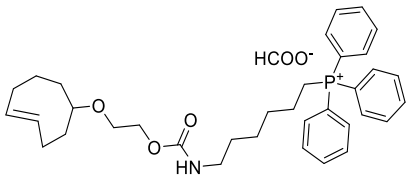
PURIFICATION: column chromatography, PE/EA 1:2 to 1:4
YIELD: 18 mg (32%)
^{1}H NMR (400 MHz, $CDCl_3$ / CD_3OD 10:1): δ 8.77 – 8.81 (m, 2H), 8.38 – 8.42 (m, 2H), 8.31 (d, *J* = 16.0, 1H), 7.52 – 7.56 (m, 2H), 7.22 (d, *J* = 16.0, 1H), 6.38 – 6.42 (m, 2H), 3.97 (t, *J* = 7.3, 4H), 2.39 (pent, *J* = 7.3, 2H).
^{13}C NMR (101 MHz, $CDCl_3$ + CD_3OD 10:1): δ 166.1, 161.0, 153.4, 150.6, 144.0, 140.2, 130.3, 123.6, 121.1, 114.4, 110.9, 51.8, 16.6.
HRMS (APCI+) calcd. for $C_{18}H_{17}N_6^+$ $[M+H]^+$: 317.1509, found 317.1510.

(*E*)-3-(4-Azetidin-1-yl)styryl)-6-(thiophen-3-yl)-1,2,4,5-tetrazine **1f**



PURIFICATION: column chromatography (DCM)
YIELD: 42 mg (75%)
^{1}H NMR (400 MHz, $CDCl_3$ + CD_3OD 10:1): δ 8.54 (dd, *J* = 3.1, 1.2, 1H), 8.24 (d, *J* = 16.0, 1H), 8.04 (dd, *J* = 5.1, 1.2, 1H), 8.54 (dd, *J* = 3.1, 1.2, 1H), 7.54 – 7.58 (m, 2H), 7.50 (dd, *J* = 5.1, 3.1, 1H), 7.23 (d, *J* = 16.0, 1H), 6.42 – 6.46 (m, 2H), 3.99 (t, *J* = 7.3, 4H), 2.42 (pent, *J* = 7.3, 2H).
^{13}C NMR (101 MHz, $CDCl_3$ + CD_3OD 10:1): δ 164.9, 160.3, 153.1, 141.7, 135.3, 129.9, 129.1, 127.4, 126.5, 124.1, 115.3, 111.0, 52.0, 16.7.
HRMS (APCI+) calcd. for $C_{17}H_{16}N_5S^+$ $[M+H]^+$: 322.1121, found 317.1122.

Synthesis of TPP-TCO



To a solution of TPP amine[2] 35.5 mg (1.25 equiv) in 500 µL of dry CH_3CN was added 20 mg NHS-TCO active ester (major equatorial isomer)[1, 3] dissolved in 500 µL of dry CH_3CN followed by addition of 28 µL (2.5 equiv) of DIPEA. The progress of the reaction was followed by HPLC/MS. After 30 min the reaction mixture was concentrated under reduced pressure and the residue purified by flash chromatography using gradient of MeOH in DCM (2 to 10%). Isolated after freeze-drying as white hygroscopic powder: 31 mg (79%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 – 7.74 (m, 10H), 7.69 (m, 6H), 5.76 – 5.35 (m, 2H), 5.24 – 5.10 (m, 1H), 4.11 (q, *J* = 5.2, 2H), 3.86 – 3.65 (m, 3H), 3.65 – 3.49 (m, 2H), 3.43 – 3.28 (m, 1H), 3.21 – 3.06 (m, 3H), 2.41 – 2.26 (m, 1H), 2.26 – 1.21 (m, 14H).
^{13}C NMR (101 MHz, $CDCl_3$) δ 156.8, 135.1, 133.9, 133.8, 132.1, 130.7, 130.1, 129.6, 119.0, 118.1, 81.1, 80.3, 66.7, 64.2, 54.1, 42.4, 40.7, 34.2, 33.5, 31.2, 30.0, 29.8, 29.5, 28.5, 26.1, 25.9, 25.7, 25.6, 22.9, 22.7, 22.7, 22.4, 18.8, 17.5, 12.2.
(ESI+) HRMS $[M]^+$ m/z calculated. for $[C_{35}H_{45}O_3NP]^+$ = 558.3132, found 558.3124.

The reaction progress of eqTCO 2a with 1c monitored by HPLC-MS and by NMR

HPLC-MS

The progress of the reaction of eqTCO **2a** with tetrazine **1c** was followed by HPLC/MS. The time dependent formation of the 4,5-dihydropyridazine intermediate **3c** and the 1,4-dihydropyridazine **3c'** was measured using pH neutral NH_4OAc since the tautomerization is sensitive to pH.[4] Monitoring at 380 nm was found most suitable due to differences in absorption maxima of **3c** and **3c'**. Formation of double peaks presumably corresponds to the formation of two different dihydropyridazine regioisomers in the IEDDA reaction.

Stock solutions: **1c**: 1 mM in DMSO, eqTCO **2a**: 50 mM in CH_3CN/H_2O = 1/1
Procedure: 4 µL of eqTCO stock solution (10 equiv, 2 mM final concentration) was mixed with 26 µL CH_3CN/H_2O = 1/1. To this solution was added 20 µL of **1c** stock solution (200 µM final concentration). This solution was further diluted with 50 µL CH_3CN/H_2O = 1/1 and injected into LC/MS (10 µL injection) at different time points (Figure S1).

Gradient: (solvent A = 20 mM NH_4OAc in water, solvent B = 10 mM NH_4OAc of 90% CH_3CN in water) 5 to 95 % B in 10 min and back to 5% B.

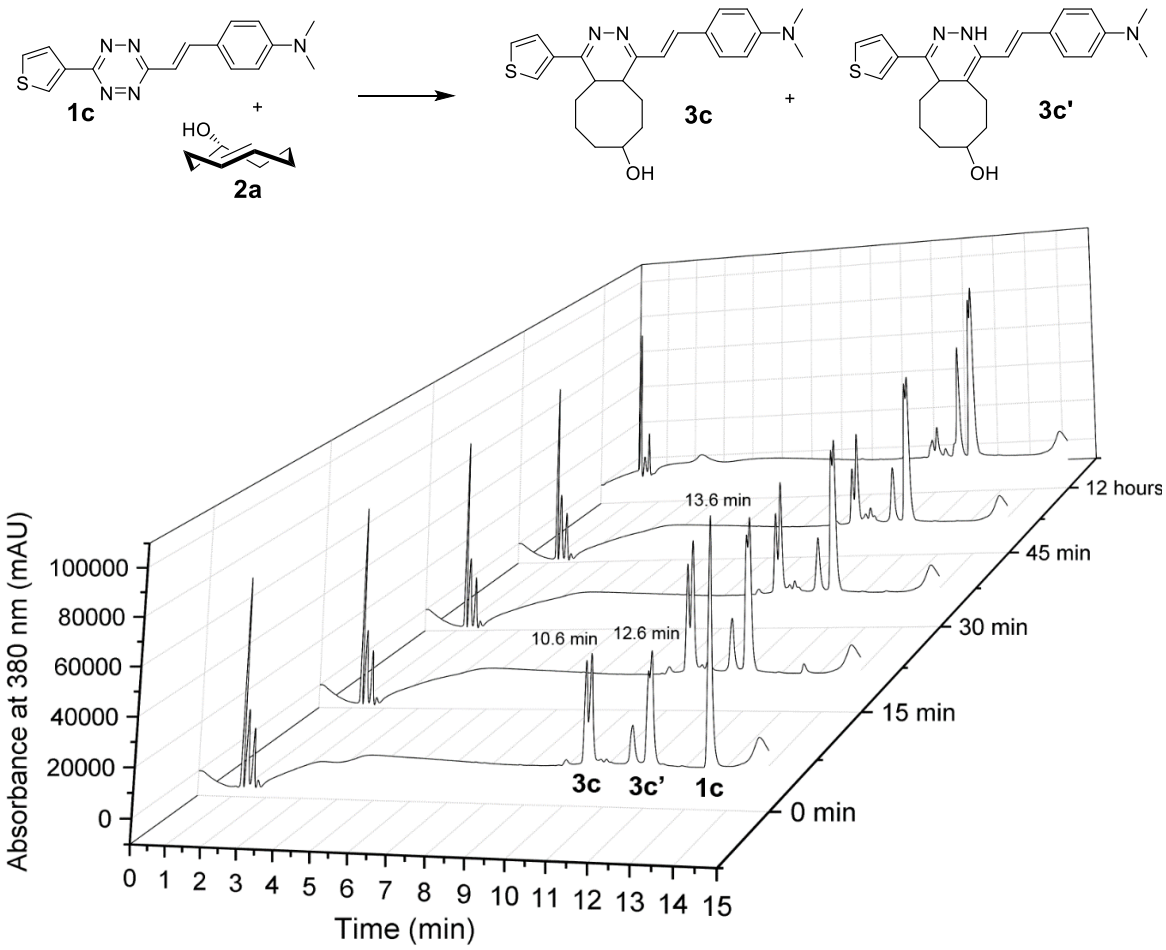


Figure S1: HPLC chromatogram showing progress of the reaction between **1c** and eqTCO **2a**. The double peak eluting at 10.6 min was ascribed to the 4,5-dihydropyridazine intermediate **3c** (observed mass 408.2), which is slowly converted to the 1,4-dihydropyridazine **3c'** (observed mass 408.2) eluting at 12.6 min. Signal at 13.6 min corresponds to the starting tetrazine **1c** (observed mass 310.1). Monitored at 380 nm. Note: We also observed formation of the oxidized pyridazine product after 12 hours. However, the product is detectable only by the MS detector and no significant signal was observed in the DAD detector.

NMR experiment

For the NMR monitoring of eqTCO reaction with compound **1c**, the studied eqTCO (ca 5 mg) was dissolved in CD_3CN (250 µL) and D_2O (250 µL) solvent mixture. After acquisition of ^1H NMR spectrum, a suspension of tetrazine **1c** (15.4 mg) in DMSO-d_6 (500 µL) was added to the solution and NMR experiments were acquired periodically, and the reaction progress was monitored. A combination of 1D (^1H and ^{13}C) experiments with 2D correlation experiments (H,H-COSY, H,C-HSQC, H,C-HMBC) was used to determine the structure of the final products. Unfortunately, the structure of the intermediates could not be confirmed unequivocally because of the complexity of the reaction mixture and difficult solubility of **1c**.

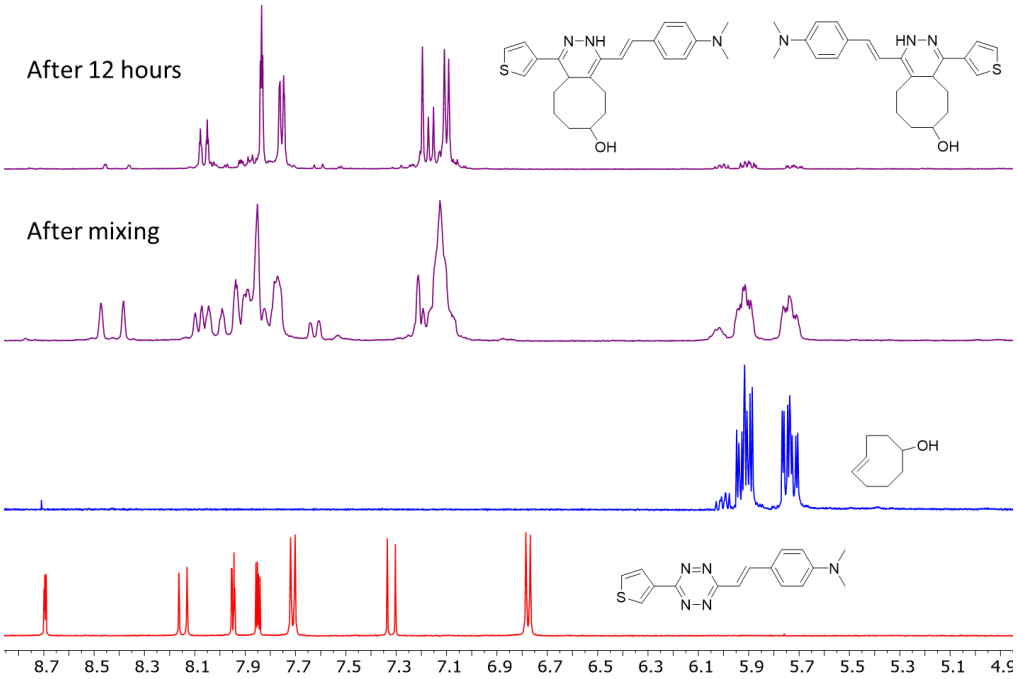


Figure S2. The double-bond and aromatic region of ^{1}H NMR spectra of **1c**, TCO **2a**, the reaction mixture immediately after mixing and after 12 hours.

Determination of second-order rate constants

Second-order rate constants of the reactions of *trans*-cyclooctenes (TCO) **2a** and **2b** with 1,2,4,5-tetrazines **1a-1f** were determined by following the decay in the concentration of the starting tetrazine over time. The concentration decrease was monitored by scanning kinetics measurements on a UV/VIS spectrophotometer. The measurements were performed in CH_3CN/H_2O 1:1 at room temperature under pseudo first-order conditions using an excess of the corresponding TCO. All measurements were performed at least three times.

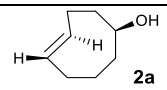
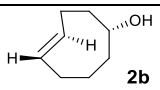
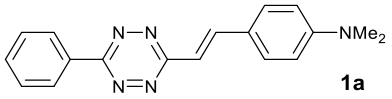
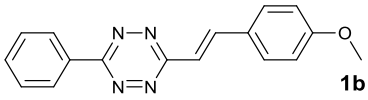
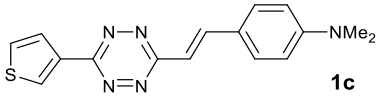
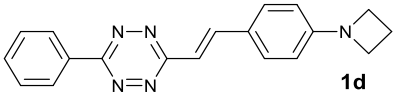
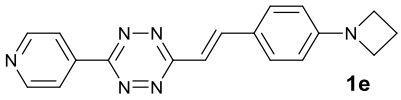
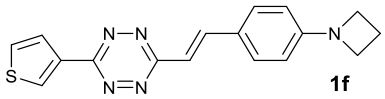
Conditions: A 50 µM solution of the respective tetrazine in CH_3CN/H_2O 1:1 containing 5% of DMSO was mixed with a 500 µM solution of the appropriate TCO in CH_3CN/H_2O 1:1. The mixture was further diluted with CH_3CN/H_2O 1:1 to give a final tetrazine concentration of 12.5 µM using 10 equiv of TCO and was immediately measured on the UV/VIS spectrophotometer.

The absorption of the mixtures was followed by scanning kinetics measurements over 4 min. The measured intensity of the absorption at the corresponding absorption maxima of the starting tetrazine (see Table S1) was plotted against time. Fitting the curves with single exponential equation ($y = y_0 + Ae^{-k/t}$) provided the observed rate constants. The second order rate constants (summarized in Table S1) were calculated by dividing the observed rate constants by the initial concentration of the TCO.

The second order rate constants of the click-reactions using tetrazine **1c** were additionally determined by measuring pseudo first order rate constants at different TCO concentrations (10 equiv., 15 equiv. and 20 equiv. of TCO **2a** and 5 equiv., 7.5 equiv. and 10 equiv. of TCO **2b**). The measurements were performed in CH_3CN/H_2O 1:1 at room temperature using a final tetrazine concentration of 12.5 µM. The observed rate constants were plotted against the concentration of the

TCO in order to obtain the second order rate constants (see Table S1) from the slope of the resulting plot.

Table S1. Absorption maxima of 1,2,4,5-tetrazines used during kinetic measurements and the corresponding second-order rate constants (in M^{-1} s^{-1}) of the reactions with TCOs **2a** and **2b**.

Tetrazine	λ_{Abs} [nm][a]	k_2 [M^{-1} s^{-1}][b]	
		 **2a** equatorial	 **2b** axial
 **1a**	430	7.8 ± 0.3	35.7 ± 0.5
 **1b**	355	5.4 ± 0.2	66.9 ± 0.8
 **1c**	425	7.9 ± 0.5[c]	27.3 ± 0.6[c]
 **1d**	415	6.8 ± 0.4	39.2 ± 0.5
 **1e**	445	n.m.[d]	136 ± 3.0
 **1f**	415	8.0 ± 1.5	30.9 ± 1.1

a) Absorption maxima of the starting tetrazines were determined in CH_3CN/H_2O 1:1 at room temperature using a 25 µM solution of the respective tetrazine, b) second-order rate constants were determined in CH_3CN/H_2O 1:1 at room temperature under pseudo first-order conditions using an excess of TCO, c) second order rate constant for derivative **1c** were additionally determined using three different TCO concentrations (10, 15 and 20 equiv. for eqTCO and 5, 7.5 and 10 equiv. of axTCO). The determined k_2 were 6.5 M^{-1} s^{-1} and 36.3 M^{-1} s^{-1} respectively, d) not measured; decay of the tetrazine was not traceable due to overlapping of the absorption maximum of the tetrazine and the absorption maximum of the corresponding click product.

Photophysical properties of the click products of tetrazines 1a-1f with equatorial TCO 2a

Conditions for the following absorption and emission measurements:
A 1 mM solution of the respective tetrazine in DMSO was mixed with a 50 mM solution of the appropriate TCO in CH_3CN/H_2O 1:1 and further diluted with CH_3CN/H_2O 1:1 to give a final tetrazine concentration of 500 µM using 2 equiv of TCO. The reaction mixtures were incubated at room temperature in the dark for 5 min and then further diluted to a tetrazine concentration of 25 µM for absorption measurements and to a concentration of 2.5 µM for emission measurements.

Determination of fluorescence quantum yields

Quantum yields of click products were measured at room temperature (22 °C) in CH_3CN/H_2O 1:1 (2.5 µM final concentration) using a 1 cm quartz cuvette. The settings were as follows: Excitation wavelength 550 nm, slit 5.0 nm; Emission 575-750 nm, increment 1.0 nm, slit 5.0 nm and data algebra formula S1c/R1c. The fluorescence quantum yields were calculated using the following equation:

$$\phi_{sample} = \phi_{ref} \frac{F_{sample}}{F_{ref}} \times \frac{(1\text{-}10^{-abs})_{ref}}{(1\text{-}10^{-abs})_{sample}} \times \frac{n^2_{sample}}{n^2_{ref}}$$

Where:

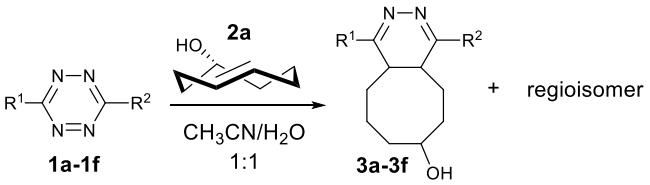
ϕ_{ref} is 0.38 (fluorescence quantum yield of nile red in MeOH)[5]

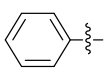
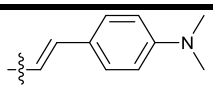
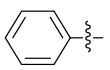
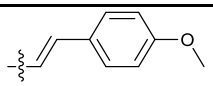
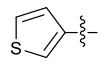
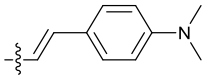
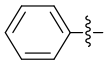
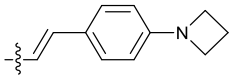
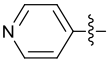
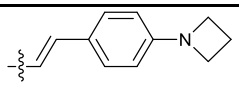
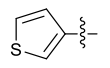
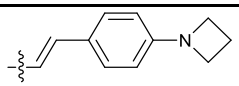
F are the integrated intensities (areas) of the standard and the sample fluorescence spectra (integrals calculated using OriginPro software)

abs is the absorbance of standard and sample at the excitation wavelength (550 nm)

n are the refractive indices for standard (MeOH: 1.327) and sample solution (CH_3CN/H_2O (1:1): 1.3478)[6]

Table S2. Photophysical properties of the click products of TCO **2a** with tetrazines **1a-f**[a].



Tetr.	R^1	R^2	$\lambda_{Abs}/\lambda_{Em}$ [b] [nm]	Stokes shift [nm]	ϕ [c]	ε_{max} ($\times 10^3$ M^{-1} cm^{-1})	Fl. intensity increase [d]
1a			545/628	83	0.005	6.4	13-fold
1b			295/-	-	-	-	-
1c			546/626	80	0.005	11.5	18-fold
1d			546/626	80	0.004	5.3	10-fold
1e			566/643	77	0.002	4.5	3-fold
1f			549/626	77	0.005	9.5	12-fold

a) All reactions were performed in CH_3CN/H_2O 1:1 at room temperature using an excess of the TCO, b) absorption and emission (at 550 nm excitation) maxima were measured in CH_3CN/H_2O 1:1 at room temperature, c) quantum yields were determined by using nile red in MeOH as standard (ϕ = 0.38), d) calculated as the integral of the fluorescence of the click product divided by the integral of the fluorescence of the starting tetrazine.

Absorption and emission spectra of the click products and starting tetrazines

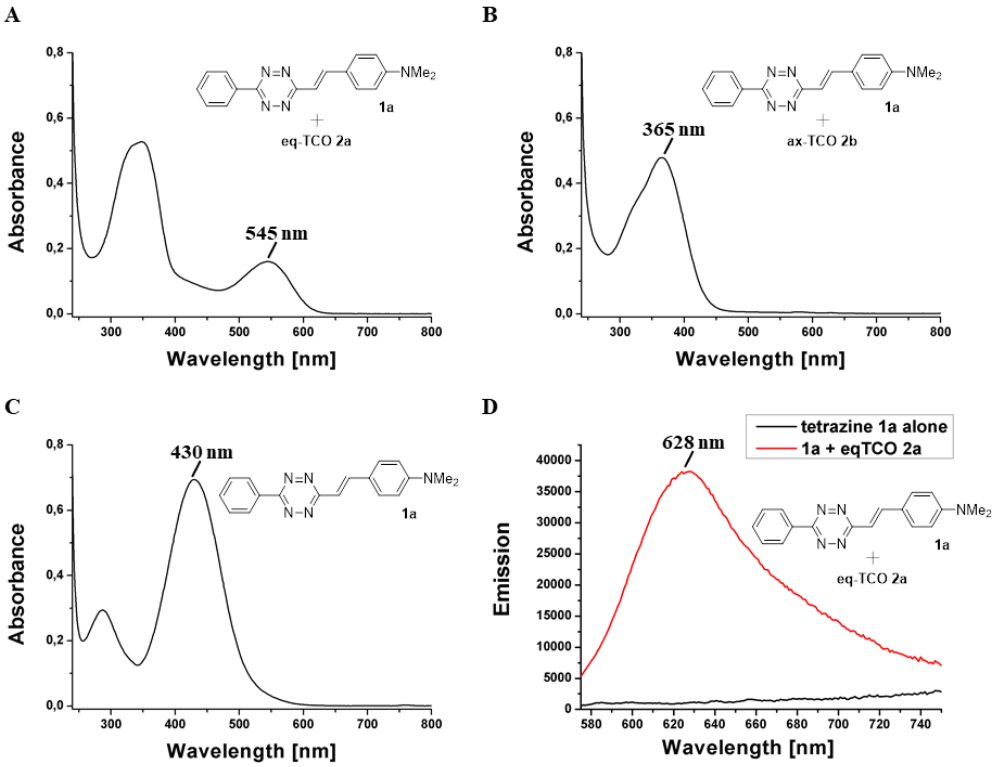


Figure S3. Absorption and emission spectra of click product formed in reaction of **1a** with **2a** (A and D), absorption spectrum of the click product of **1a** with axial TCO **2b** (B) and absorption and emission spectra of **1a** (C and D). All spectra were measured in CH_3CN/H_2O 1:1 at room temperature using a tetrazine concentration of 25 µM for absorption measurements and 2.5 µM for emission measurements.

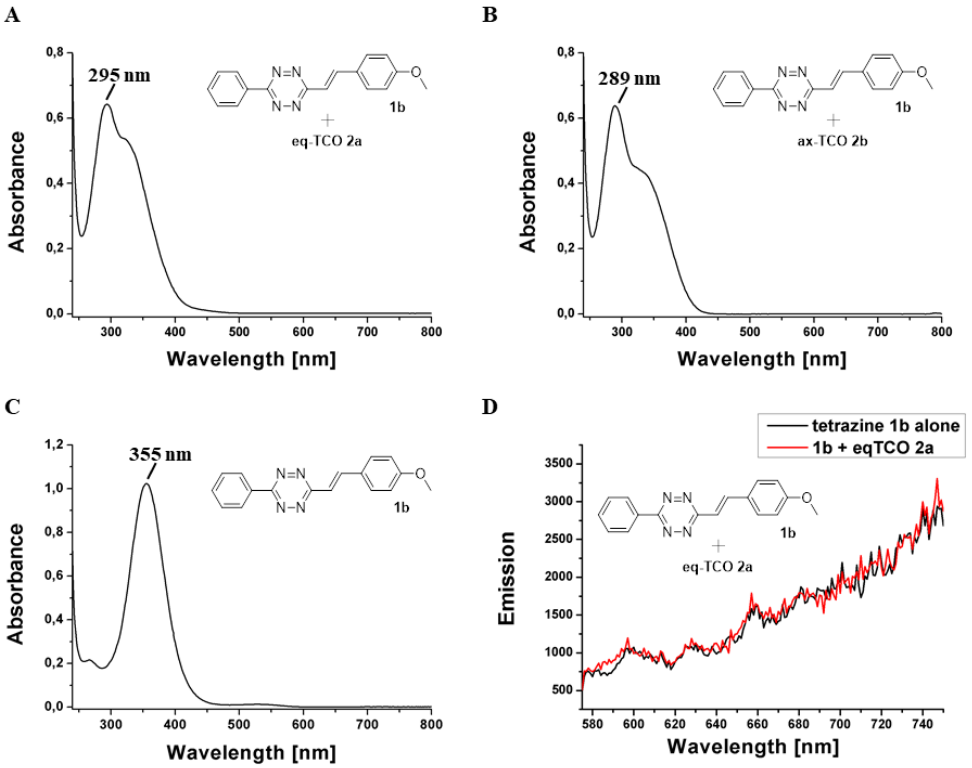


Figure S4. Absorption and emission spectra of click product formed in reaction of **1b** with **2a** (A and D), absorption spectrum of the click product of **1b** with axial TCO **2b** (B) and absorption and emission spectra of **1b** (C and D). All spectra

were measured in CH_3CN/H_2O 1:1 at room temperature using a tetrazine concentration of 25 µM for absorption measurements and 2.5 µM for emission measurements.

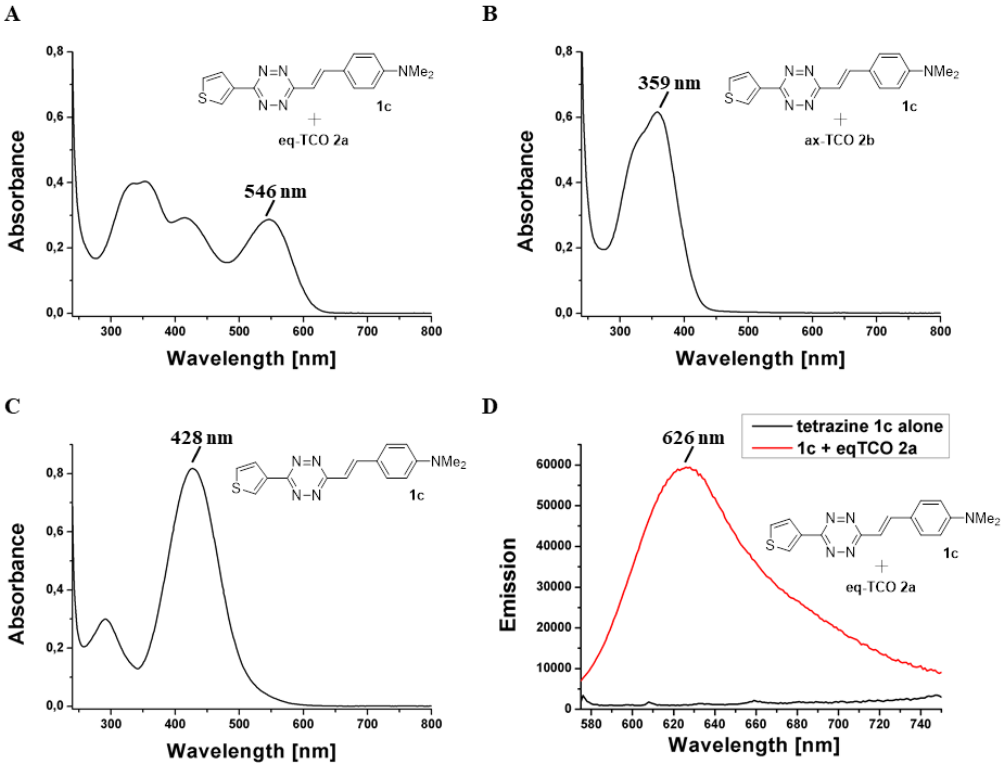


Figure S5. Absorption and emission spectra of click product formed in reaction of **1c** with **2a** (A and D), absorption spectrum of the click product of **1c** with axial TCO **2b** (B) and absorption and emission spectrum of **1c** (C and D). All spectra were measured in CH_3CN/H_2O 1:1 at room temperature using a tetrazine concentration of 25 µM for absorption measurements and 2.5 µM for emission measurements.

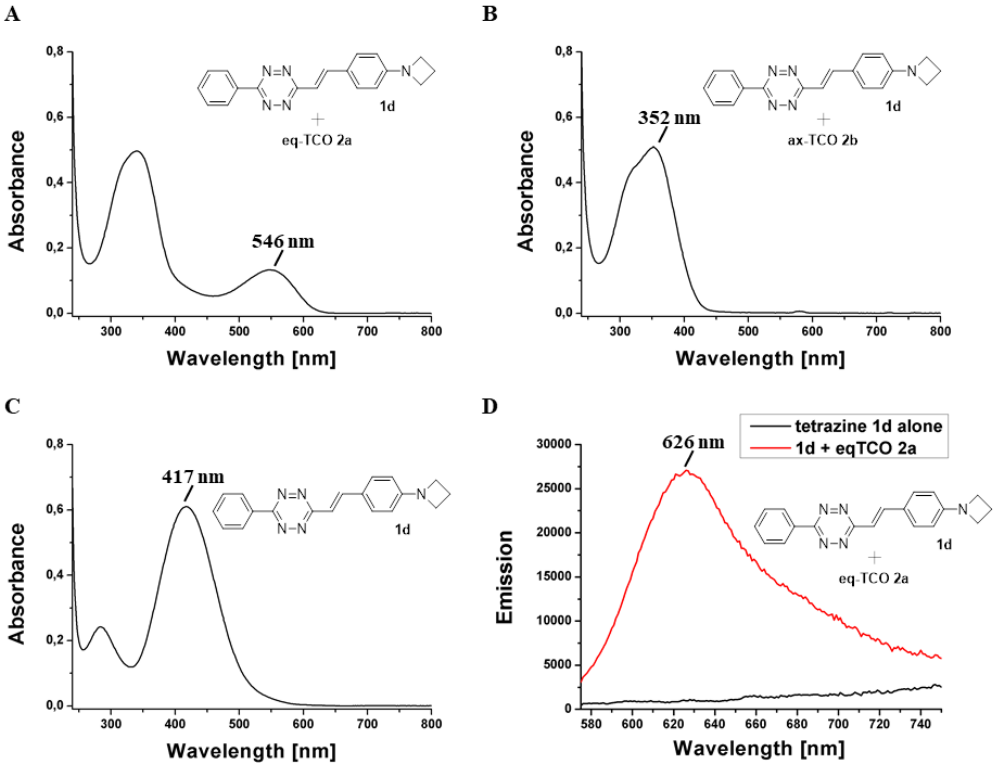


Figure S6. Absorption and emission spectra of click product formed in reaction of **1d** with **2a** (A and D), absorption spectrum of the click product of **1d** with axial TCO **2b** (B) and absorption and emission spectrum of **1d** (C and D). All spectra

were measured in CH_3CN/H_2O 1:1 at room temperature using a tetrazine concentration of 25 µM for absorption measurements and 2.5 µM for emission measurements.

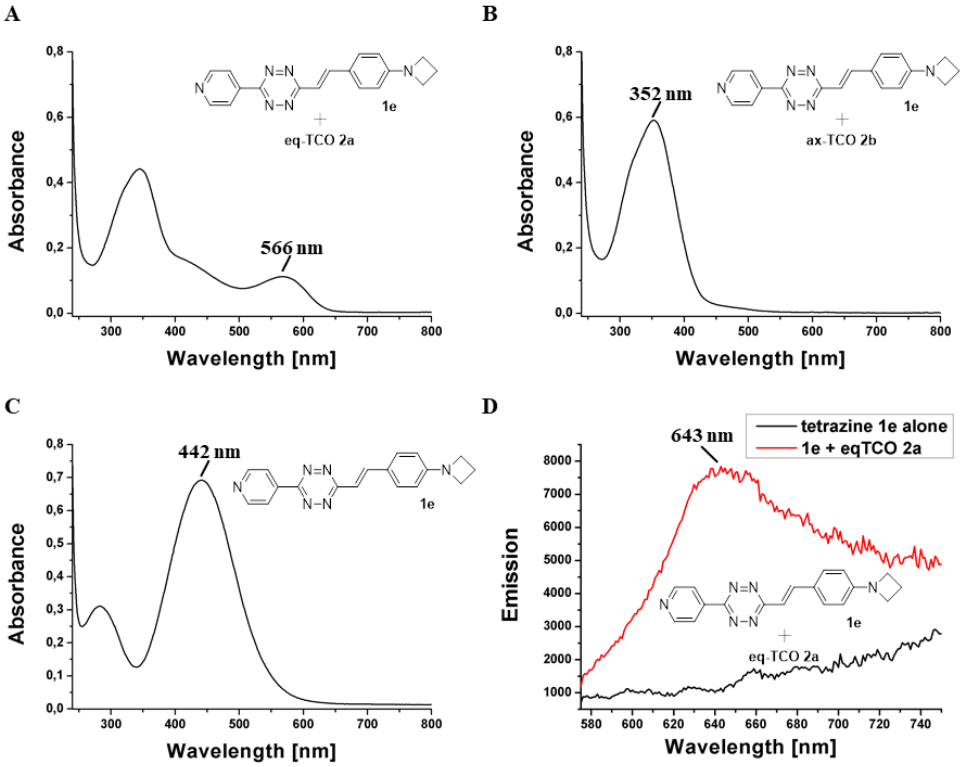


Figure S7. Absorption and emission spectra of click product formed in reaction of **1e** with **2a** (A and D), absorption spectrum of the click product of **1e** with axial TCO **2b** (B) and absorption and emission spectra of **1e** (C and D). All spectra were measured in CH_3CN/H_2O 1:1 at room temperature using a tetrazine concentration of 25 µM for absorption measurements and 2.5 µM for emission measurements.

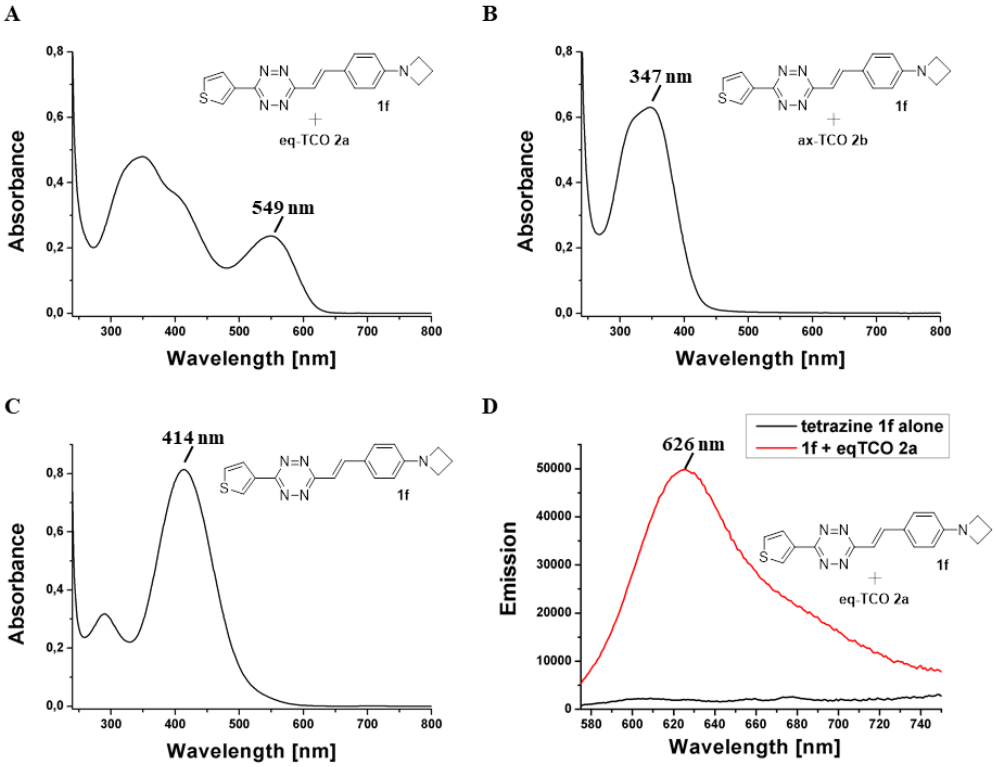


Figure S8. Absorption and emission spectra of click product formed in reaction of **1f** with **2a** (A and D), absorption spectrum of the click product of **1f** with axial TCO **2b** (B) and absorption and emission spectra of **1f** (C and D). All spectra were

measured in CH_3CN/H_2O 1:1 at room temperature using a tetrazine concentration of 25 µM for absorption measurements and 2.5 µM for emission measurements.

The fluorescence decay of the thiophene containing click products 3c and 3f over time

Conditions for the emission measurements:
A 1 mM solution of the respective tetrazine in DMSO was mixed with a 50 mM solution of TCO **2a** in CH_3CN/H_2O 1:1 and further diluted with CH_3CN/H_2O 1:1 to give a final tetrazine concentration of 500 µM using 2 equiv of TCO. The reaction mixtures were incubated at room temperature in the dark for 5 min and then further diluted to a tetrazine concentration of 2.5 µM for the emission measurements. The diluted solutions were measured at indicated time points (Figure S9 and S10). The settings were as follows: Excitation wavelength 550 nm, slit 5.0 nm; Emission 575-750 nm, increment 1.0 nm, slit 5.0 nm and data algebra formula S1c/R1c.
Or: Excitation wavelength 350 nm, slit 5.0 nm; Emission 400-680 nm, increment 1.0 nm, slit 5.0 nm and data algebra formula S1c/R1c.

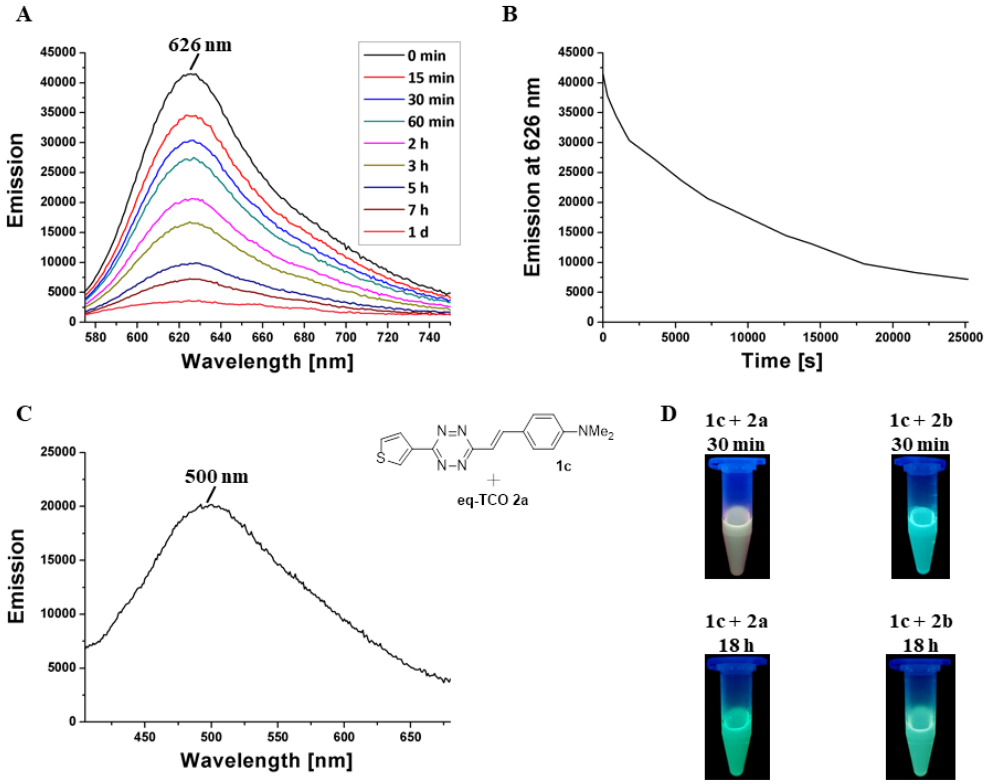


Figure S9. Emission spectra of click product formed in reaction of **1c** with **2a** excited at 550 nm at different time points (A), corresponding decay of the emission maximum at 626 nm over time (B), emission spectrum of click product formed in reaction of **1c** with **2a** after 1 d of incubation at 350 nm excitation (C) and fluorescence pictures of click products formed in the reactions of **1c** with **2a** and **2b** taken after 30 min and 18 h respectively (captured under UV-Vis lamp, 354 nm) (D). All spectra were measured in CH_3CN/H_2O 1:1 at room temperature using a tetrazine concentration of 2.5 µM.

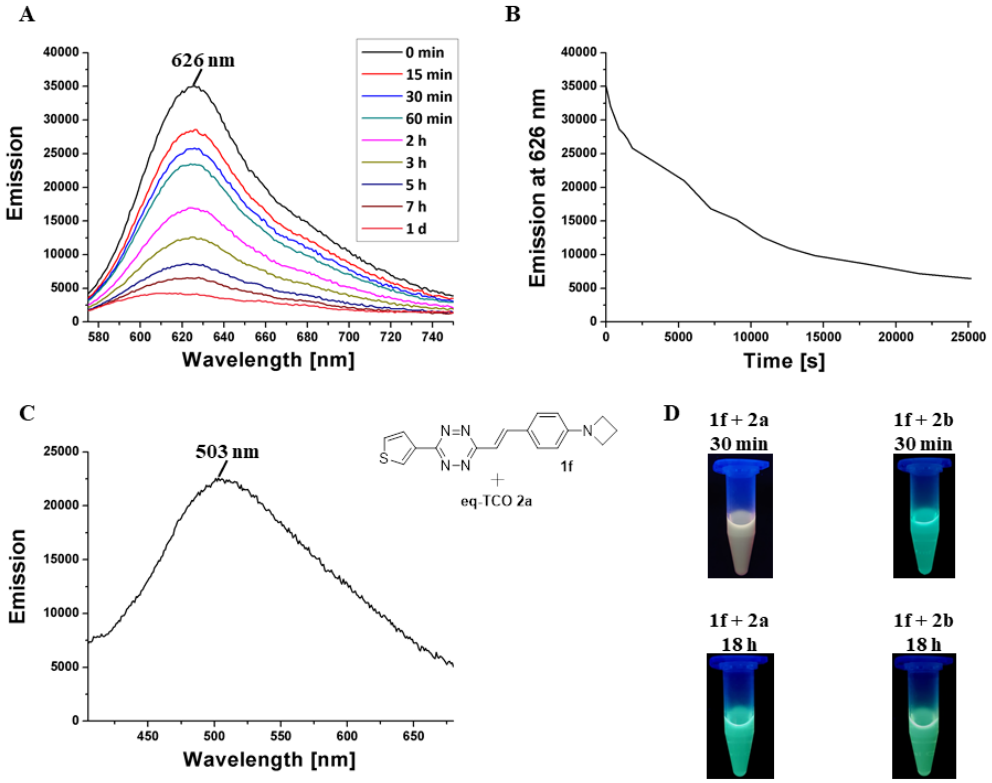


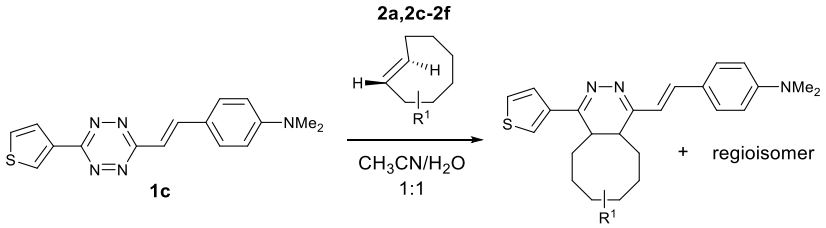
Figure S10. Emission spectra of click product formed in reaction of **1f** with **2a** excited at 550 nm at different time points (A), corresponding decay of the emission maximum at 626 nm over time (B), emission spectrum of click product formed in reaction of **1f** with **2a** after 1 d of incubation at 350 nm excitation (C) and fluorescence pictures of click products formed in the reactions of **1f** with **2a** and **2b** taken after 30 min and 18 h respectively (captured under UV-Vis lamp, 354 nm) (D). All spectra were measured in CH_3CN/H_2O 1:1 at room temperature using a tetrazine concentration of 2.5 µM.

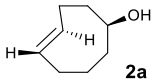
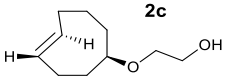
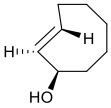
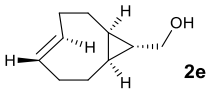
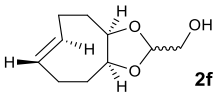
The fluorescence comparison of the thiophene containing tetrazines 1c and 1f with different TCOs

Conditions for the absorption and emission measurements:

A 1 mM solution of the respective tetrazine in DMSO was mixed with a 50 mM solution of the appropriate TCO in CH_3CN/H_2O 1:1 and further diluted with CH_3CN/H_2O 1:1 to give a final tetrazine concentration of 500 µM using 2 equiv of TCO. The reaction mixtures were incubated at room temperature in the dark for 5 or 30 min and then further diluted to a tetrazine concentration of 25 µM for absorption measurements and 2.5 µM for emission measurements. The settings for emission measurements were as follows: Excitation wavelength 550 nm, slit 5.0 nm; Emission 575-750 nm, increment 1.0 nm, slit 5.0 nm and data algebra formula S1c/R1c. Quantum yields were determined by using nile red in MeOH as standard (ϕ = 0.38).

Table S3. Photophysical properties of the click products of tetrazine **1c** with various TCOs.



TCO	$\lambda_{Abs}/\lambda_{Em}$ [a] [nm]	Stokes shift [nm]	ϕ	ε_{max} ($\times 10^3$ M^{-1} cm^{-1})	Fl. intensity increase [b]
 **2a** equatorial	546/626	80	0.005	11.5	18-fold
2c 	547/627	80	0.004	11.5	13-fold
 **2d**	550/627	77	0.004	14.4	14-fold
 **2e**	547/631	84	0.003	12.2	11-fold
 **2f**	558/638	80	0.002	7.8	6-fold

a) Absorption and emission maxima were measured in CH_3CN/H_2O 1:1 at room temperature, b) calculated as the integral of the fluorescence of the click product divided by the integral of the fluorescence of the starting tetrazine.

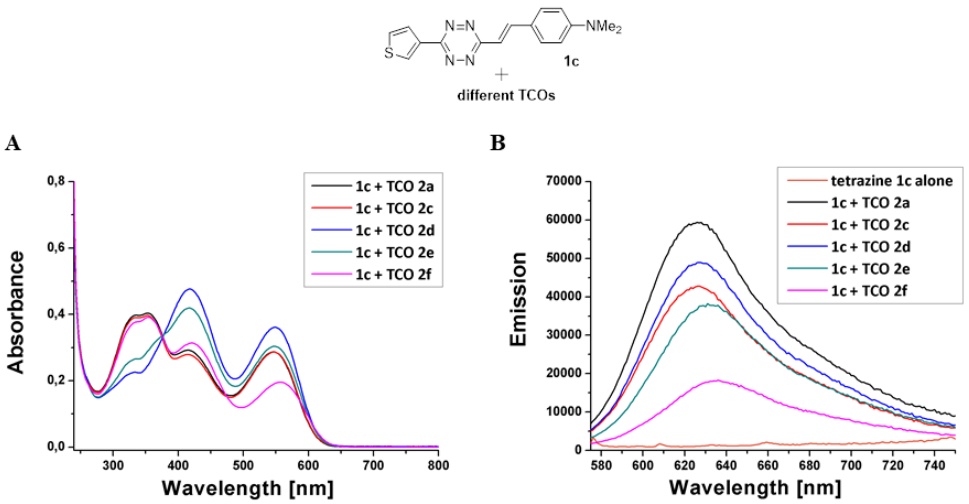
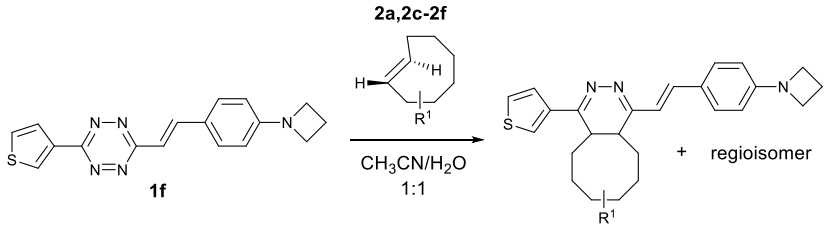
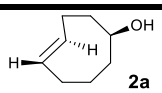
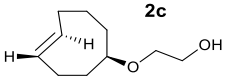
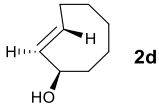
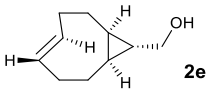
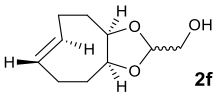


Figure S11. Absorption (A) and emission (B) spectra of the click products of **1c** with various TCOs. The spectra were measured after 5 min (for click reactions with TCO **2a, 2e** and **2f**) or after 30 min (for click reactions with TCO **2c** and **2d**) of incubation.

Table S4. Photophysical properties of the click products of tetrazine **1f** with various TCOs.



TCO	$\lambda_{Abs}/\lambda_{Em}$ [b] [nm]	Stokes shift [nm]	ϕ [c]	ε_{max} ($\times 10^3$ M^{-1} cm^{-1})	Fl. intensity increase [d]
 **2a** equatorial	549/626	77	0.005	9.5	12-fold
 **2c**	550/627	77	0.004	9.5	9-fold
 **2d**	550/627	77	0.003	11.6	9-fold
 **2e**	550/631	81	0.003	10.1	8-fold
 **2f**	551/631	80	0.002	6.4	4-fold

a) Absorption and emission maxima were measured in CH_3CN/H_2O 1:1 at room temperature, b) calculated as the integral of the fluorescence of the click product divided by the integral of the fluorescence of the starting tetrazine.

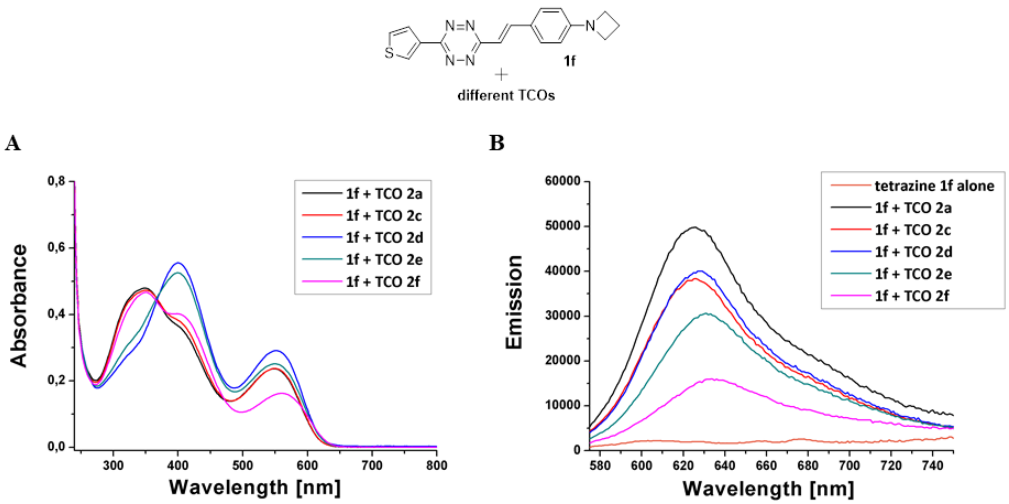


Figure S12. Absorption (A) and emission (B) spectra of the click products of **1f** with various TCOs. The spectra were measured after 5 min (for click reactions with TCO **2a, 2e** and **2f**) or after 30 min (for click reactions with TCO **2c** and **2d**) of incubation.

Cell labeling experiments

Preparation of ConA-TCO

100 µl of 5 mg/ml solution of concanavalin A (4.7 nmol of tetramer) in 1 M NaCl, 50 mM HEPES-NaOH (pH 8.3), 3 mM $CaCl_2$, 3 mM $MnCl_2$ was combined with 2.3 µL of 10 mM NHS TCO ester[1, 3] (5× molar excess of active ester dissolved in dry DMSO). Reaction was incubated at room temperature for 1 h with constant shaking. After one hour, 10 µL of 1 M Tris-HCl (pH 6.8) was added to neutralize the remaining NHS ester and incubated for another 10 min. at room temperature. TCO excess was removed by desalting using Zeba (Thermo) spin columns into 1 M NaCl, 20 mM Tris-HCl (pH 6.8), 3 mM $CaCl_2$, 3 mM $MnCl_2$.

Cell experiments

HeLa cells were maintained in high glucose DMEM (Sigma) supplemented with 10% FBS (Thermo) and 0.1 mg/mL of penicillin-streptomycin (Sigma) at 37 °C/5% CO_2. One day before the experiment 2×10^4 cells were seeded at the 96 well plate cultivation dishes with a coverglass in the bottom (CellVis).

Cells were incubated with the TCO-TPP or ConA-TCO compounds in complete media for the indicated time points at 37 °C. Cells were then washed three times with 100 µL of Leibovitz's L15 media (Thermo) containing 1% BSA. After the last wash, cells were incubated with 100 µL of tetrazine (**1c**)-containing Leibovitz's L15 (1% BSA) in final concentration 5 µM with the addition of either 0.5 µM DRAQ 5 (Thermo), or 10 nM Mitotracker deep red (Thermo).

Microscopy

Pictures of live cells were taken using Leica TCS SP5 Tandem confocal microscope equipped with a HCX PL APO 63x/1.30 GLYC CORR. 37 °C objective. Excitation for the click products was 561 nm. Emission was collected sequentially using Hyd detector in BrightR mode, with a AOBS window set to λ = 568-620 nm. DRAQ5 for nuclei staining was excited with a 633 nm laser and collected in a 643-703 nm window. Mitotracker: excitation 633 nm, emission window 643-703 nm. Brightness of the raw images was adjusted using Adobe Photoshop.

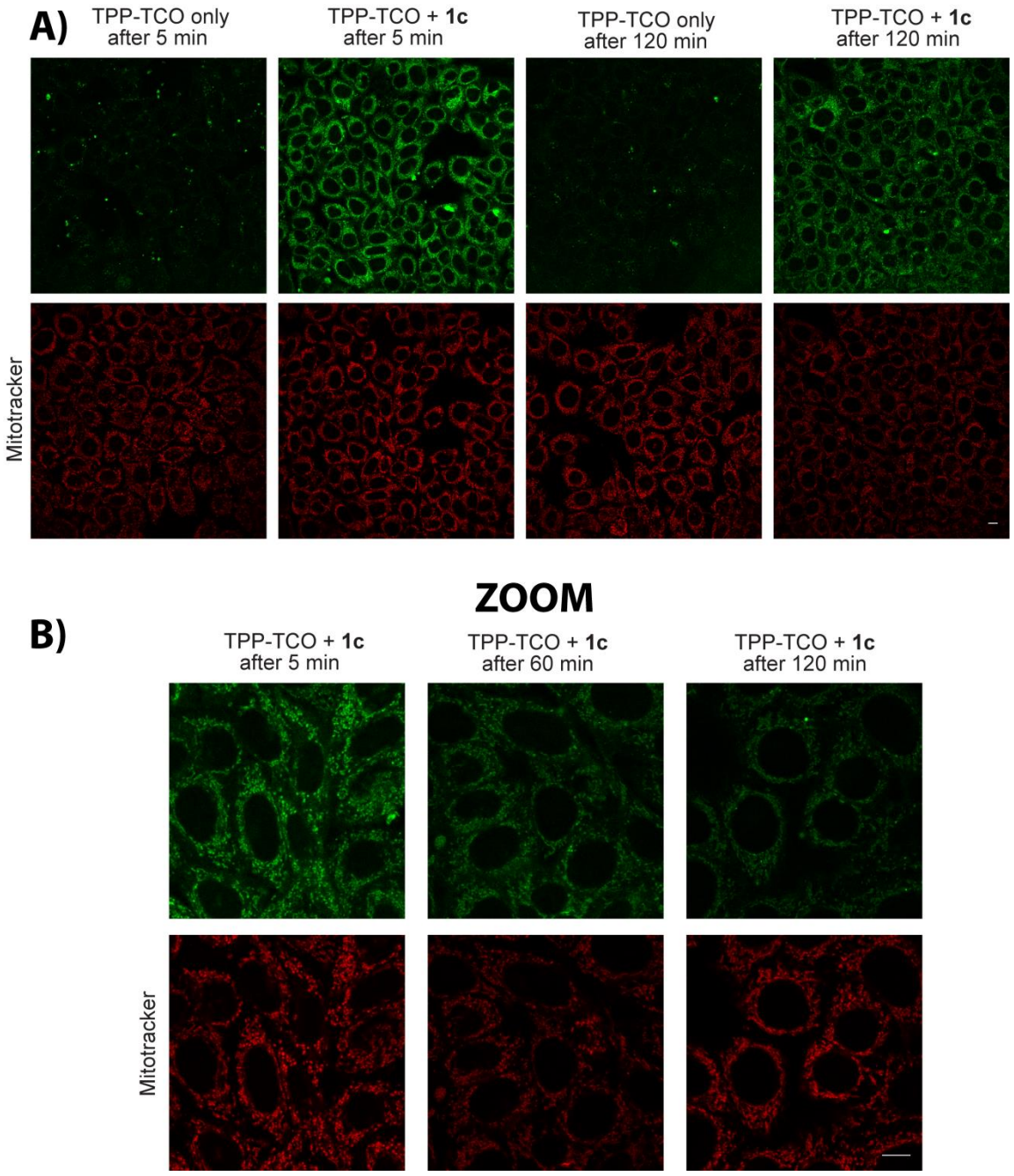


Figure S13. HeLa cells were incubated for 15 min with 5 µM TPP-TCO in Leibowitz L15 media containing 1% BSA + 10 nM Mitotracker deep red. Washed 3× with Leibowitz L15 media with 1% BSA. Incubated for 5-120 min with 5 µM (final) concentration of **1c** and imaged at indicated time points using Leica SP5 confocal microscope equipped with 63× glycerol immersion objective. For the click products, a λ = 561 nm laser was used for excitation (intensity 50%), emission was collected in a λ = 568-620 nm window. Mitotracker deep red was excited with laser λ = 633 nm, intensity 20%, emission was collected in a λ = 643-703 nm window. Scale bar 10 µm.

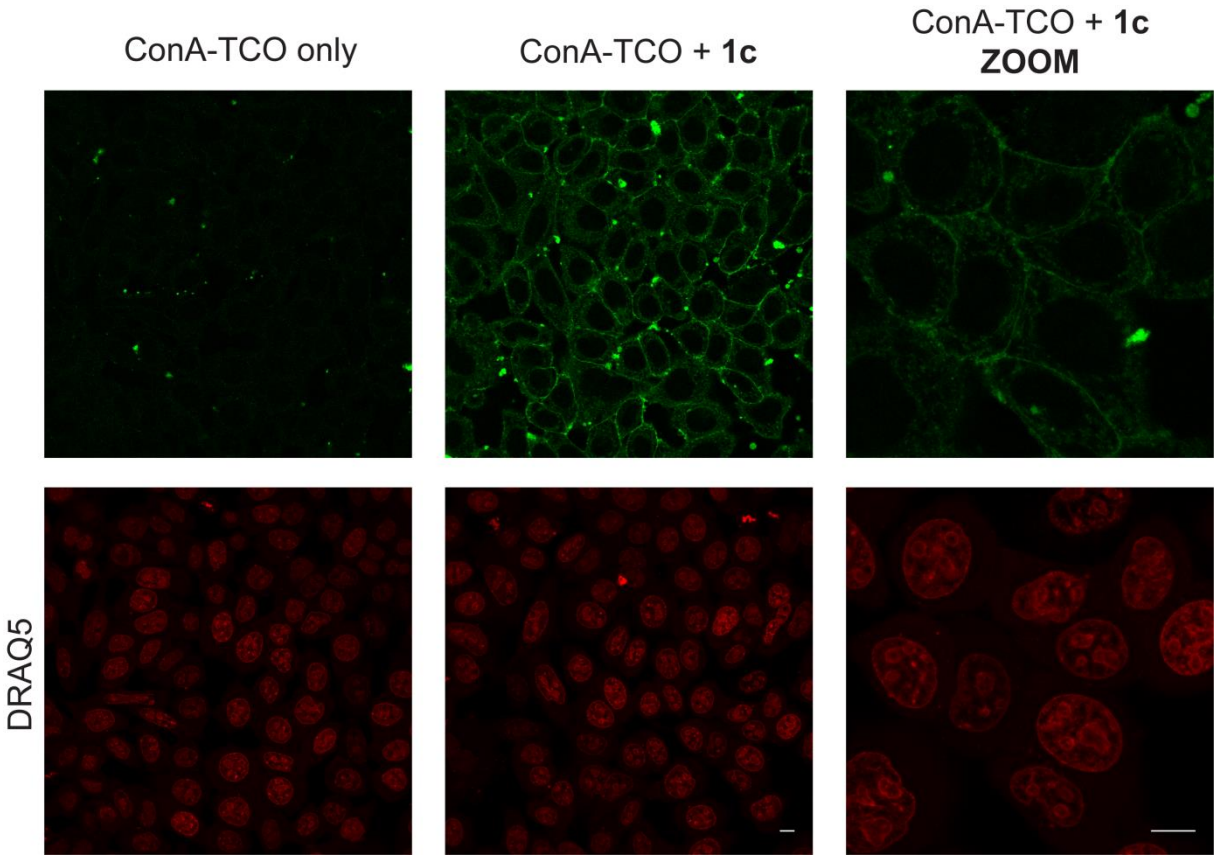


Figure S14. HeLa cells were incubated for 15 min with 1/10 dilution of ConA-TCO conjugate (0.5 mg/mL final concentration) in Leibowitz L15 media with 1% BSA + 0.5 µM DRAQ5. Washed 3× with Leibowitz L15 media with 1% BSA. Incubated for 1 h with 5 µM (final) concentration of **1c** and imaged on Leica SP5 confocal microscope, 63× glycerol immersion objective, excitation laser λ = 561 nm, intensity 50% emission λ = 568-620 nm window. DRAQ5 excitation laser λ = 633 nm, intensity 25%, emission λ = 643-703 nm window. Scale bar 10 µm.

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[2] H. Ma, B. Song, Y. X. Wang, C. L. Liu, X. Wang, J. L. Yuan, *Dyes Pigments* **2017**, *140*, 407-416.

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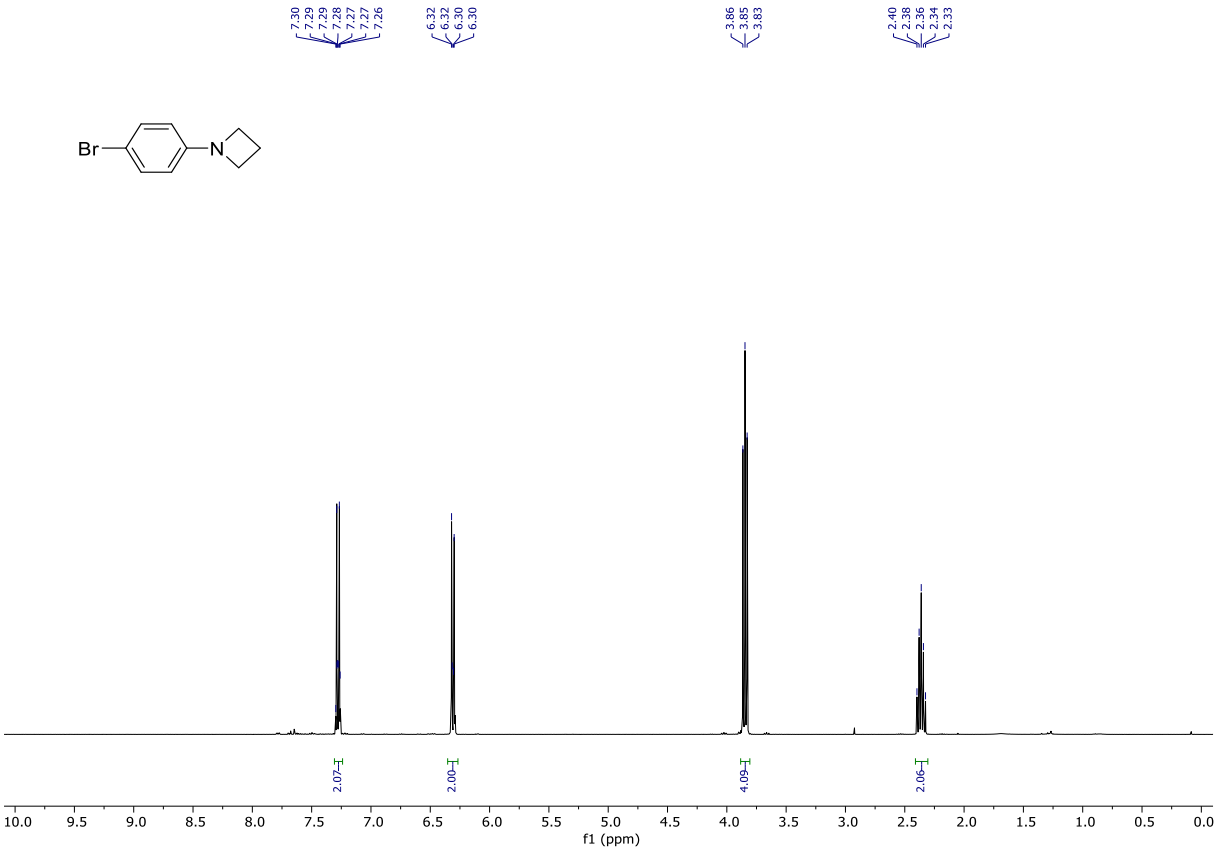
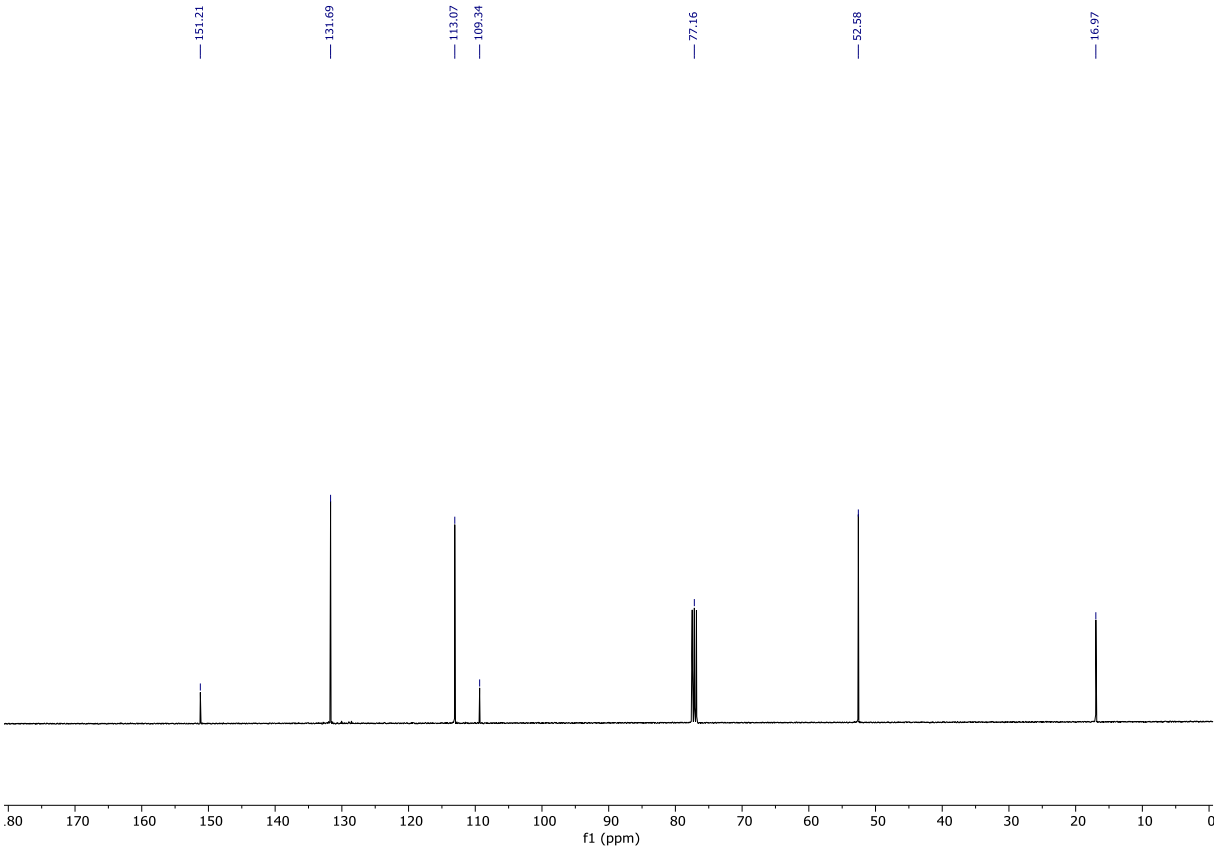
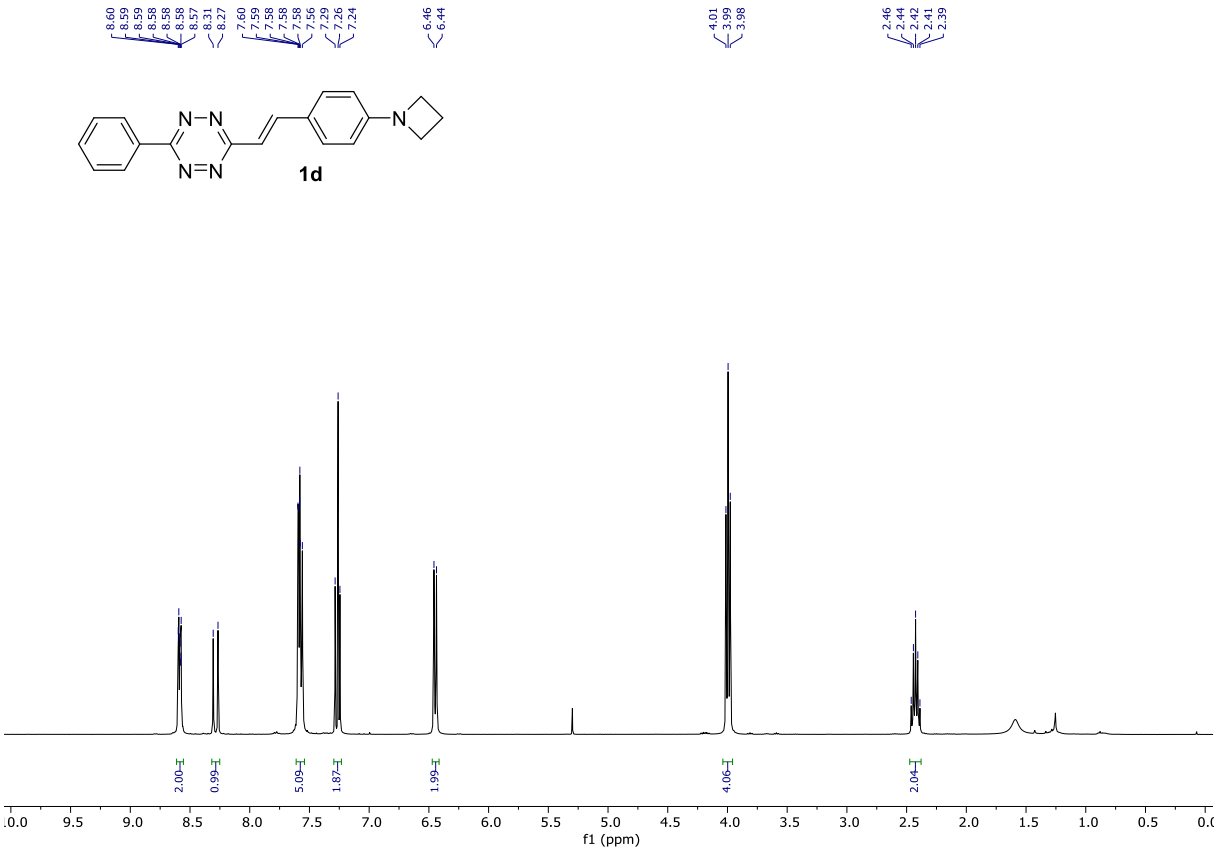
[6] J. E. Bertie, Z. D. Lan, *Journal of Physical Chemistry B* **1997**, *101*, 4111-4119.

Copies of NMR spectra



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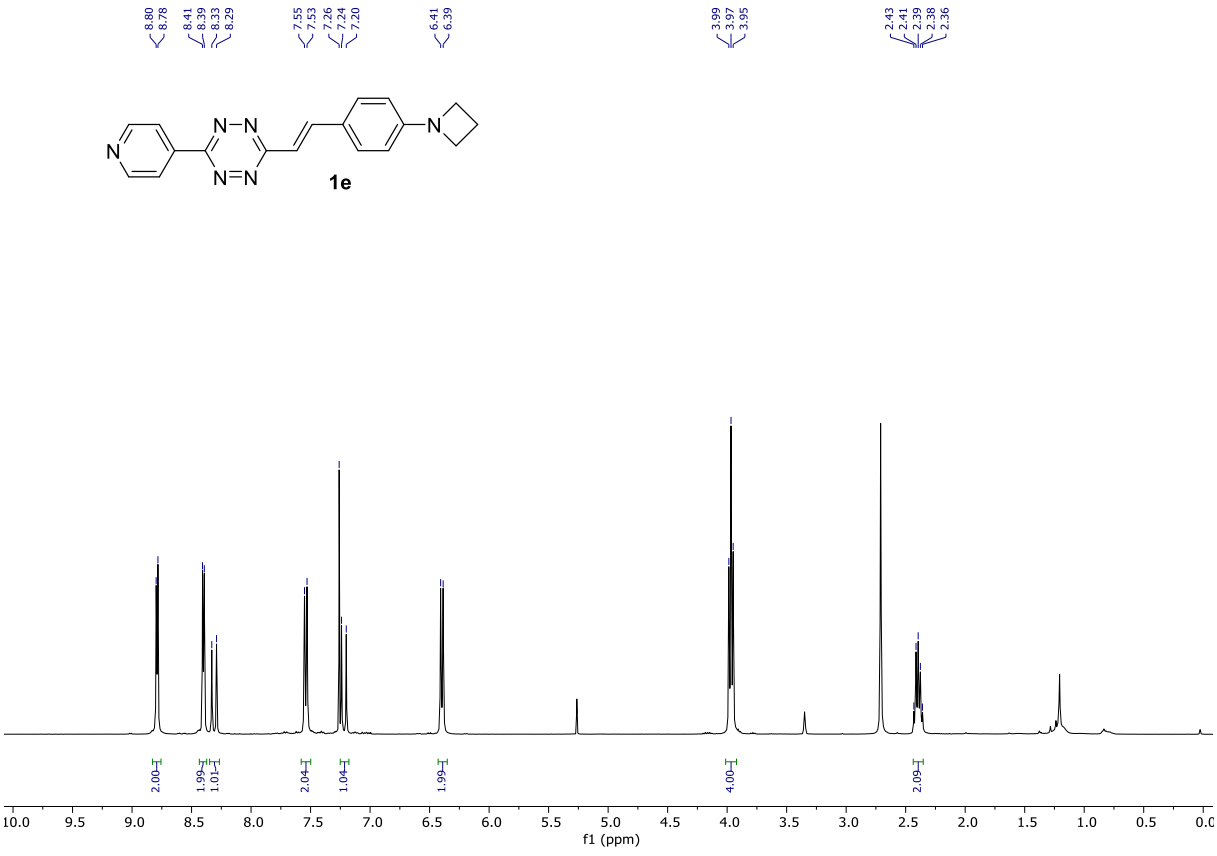
8.59
8.59
8.58
8.58
8.58
8.57
8.31
8.27
7.60
7.59
7.58
7.58
7.58
7.56
7.29
7.26
7.24
6.46
6.44
4.01
3.99
3.98
2.46
2.44
2.42
2.41
2.39
N
N
N
N
1d
2.00
0.99
5.09
1.87
1.99
4.06
2.04
f1 (ppm)
165.43
162.57
153.10
142.00
132.42
132.21
129.94
129.31
127.71
124.17
115.38
111.10
77.16
52.03
16.79
f1 (ppm)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8.41
8.39
8.33
8.29
7.55
7.53
7.26
7.24
7.20
6.41
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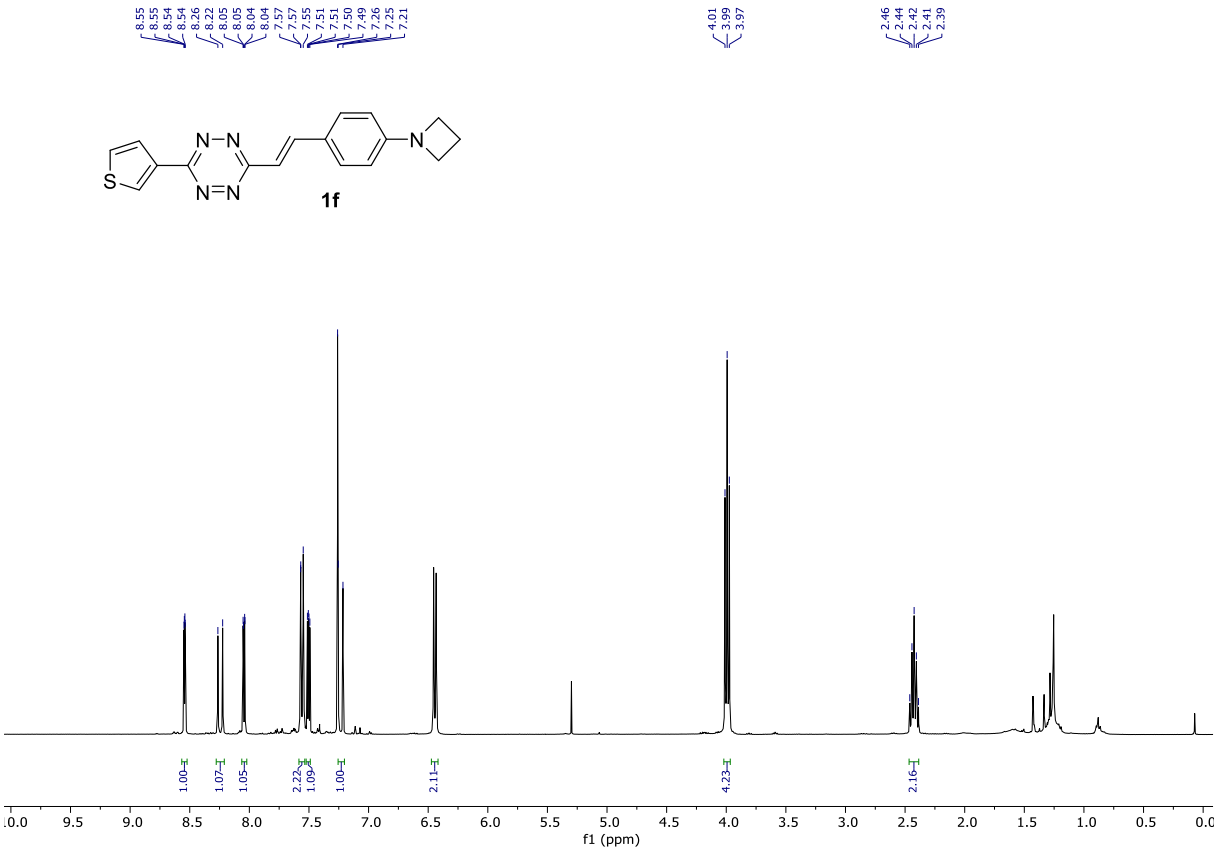
3.99
3.97
3.95
2.43
2.41
2.39
2.38
2.36
N
N
N
N
N
1e
2.00
1.99
1.01
2.04
1.04
1.99
4.00
2.09
f1 (ppm)
166.11
160.95
153.35
150.55
144.00
140.23
130.32
123.64
121.12
114.35
110.93
77.16
51.83
16.63
f1 (ppm)


8.55
8.55
8.54
8.54
8.26
8.22
8.05
8.05
8.04
8.04
7.57
7.57
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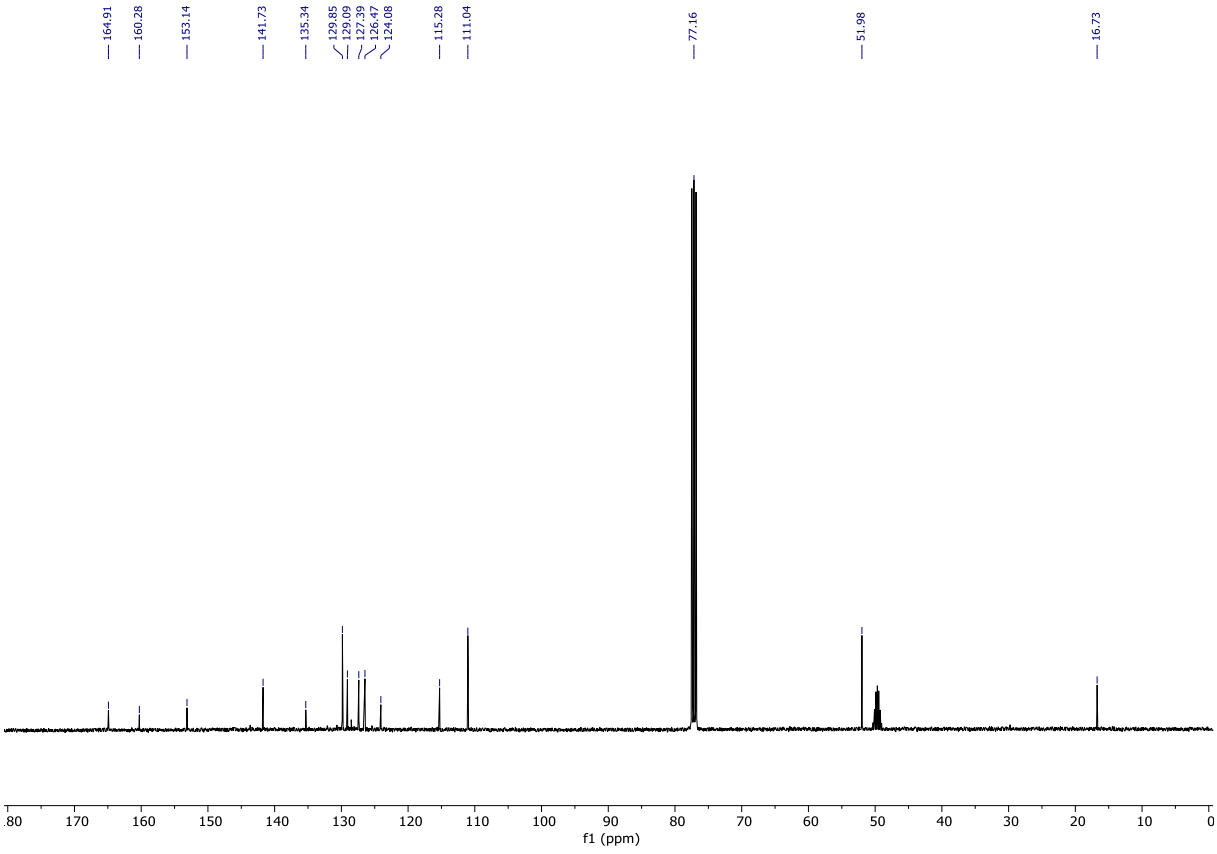
7.51
7.51
7.50
7.49
7.26
7.25
7.21
4.01
3.99
3.97
2.46
2.44
2.42
2.41
2.39
1f
1.00
1.07
1.05
2.22
1.09
1.00
2.11
4.23
2.16
f1 (ppm)


164.91
160.28
153.14
141.73
135.34
129.85
129.09
127.39
126.47
124.08
115.28
111.04
77.16
51.98
16.73
f1 (ppm)

TPP-TCO (equatorial)