Electronic Supplementary Information for

Sterically shielded tetrazoles for a fluorogenic photoclick reaction: tuning cycloaddition rate and product fluorescence

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Buffalo, New York 14260-3000, United States

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Supplemental Figures

Tetrazole **2**:

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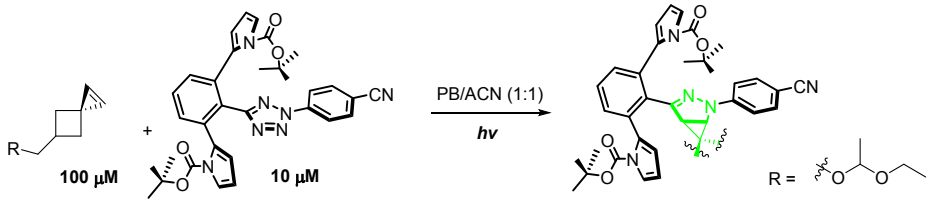

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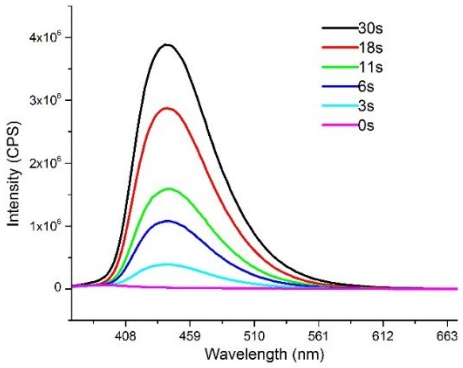

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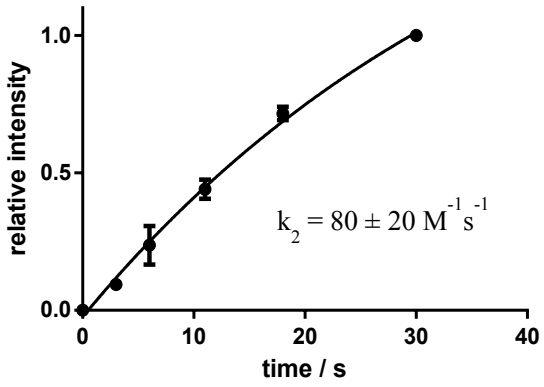

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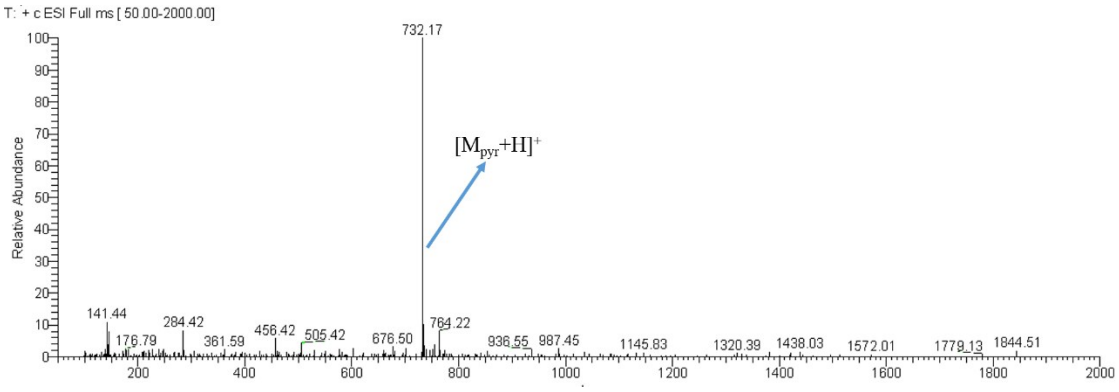

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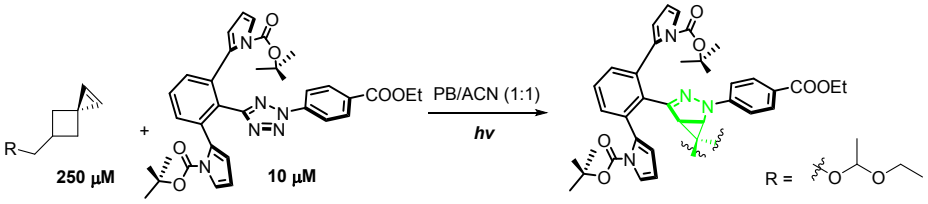

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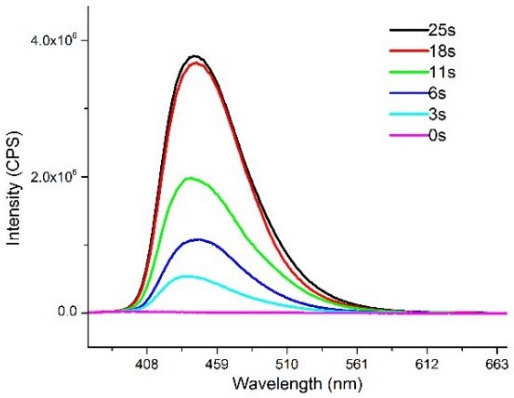

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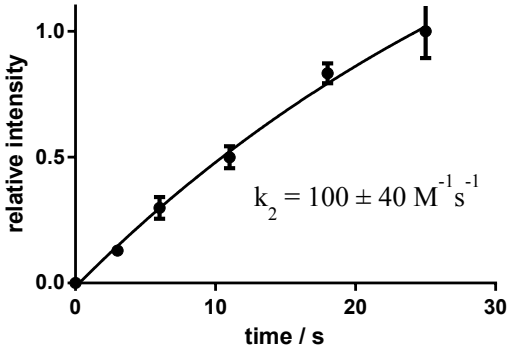

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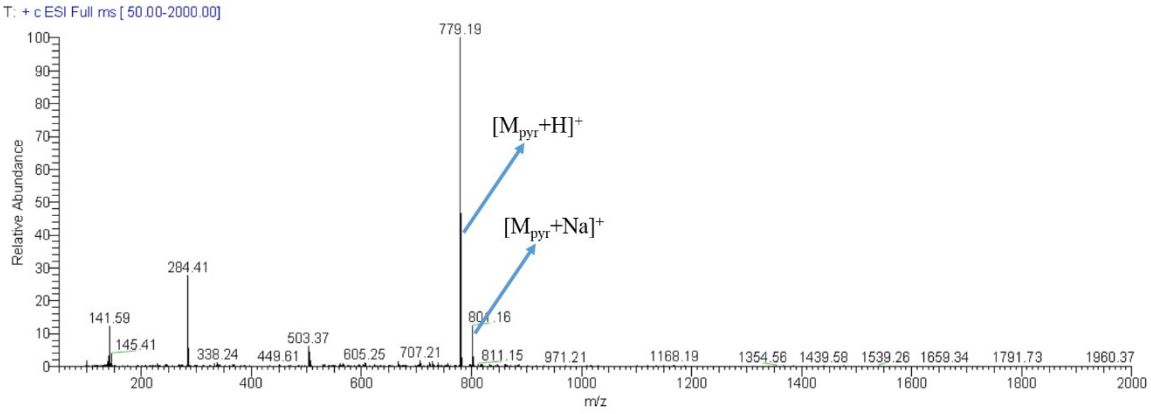

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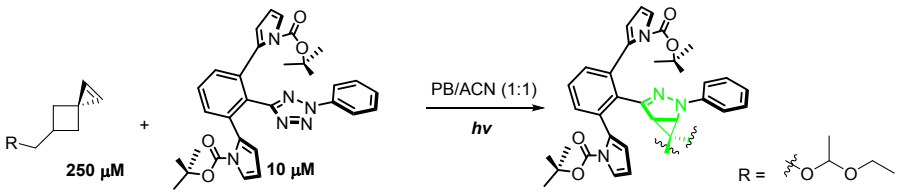

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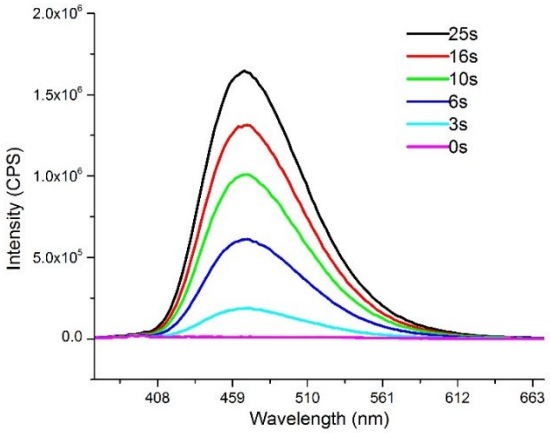

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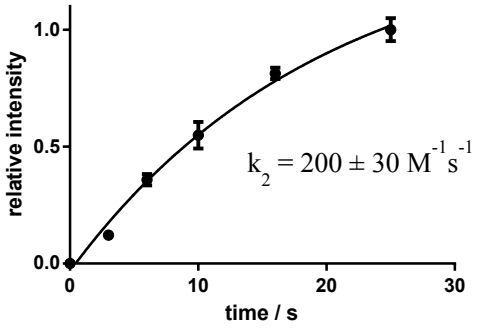

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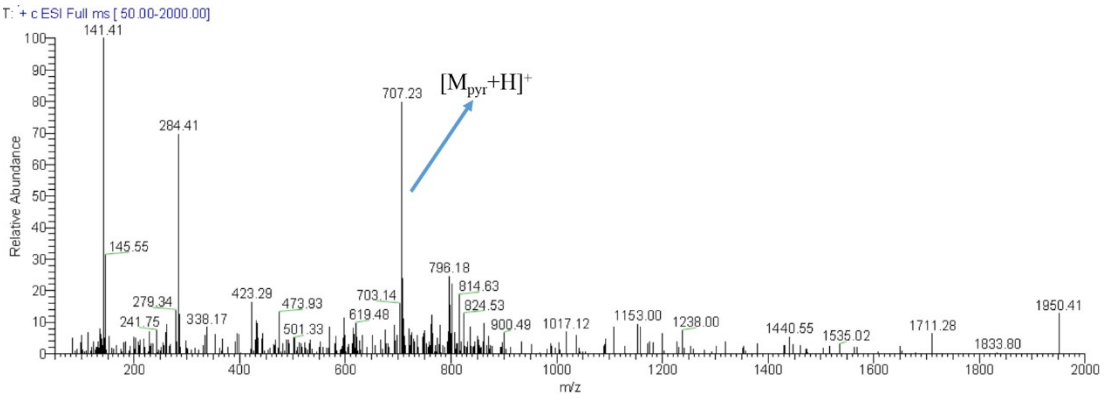

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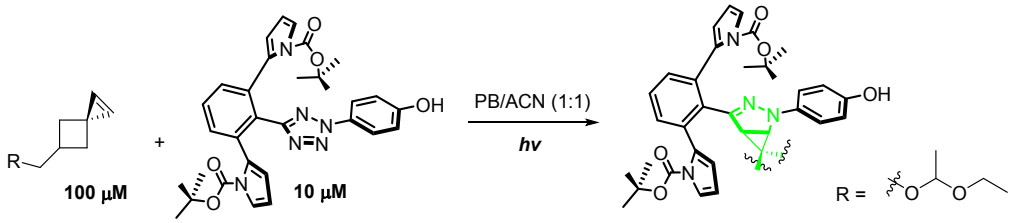

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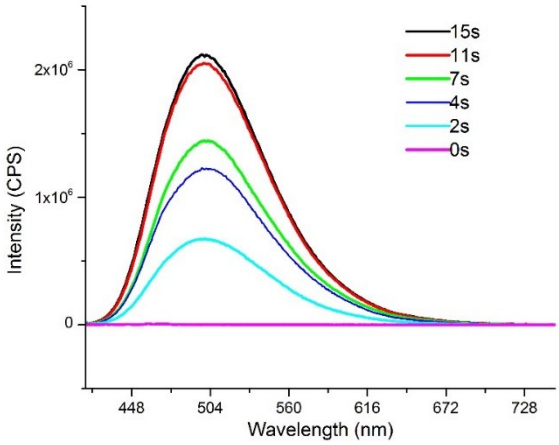

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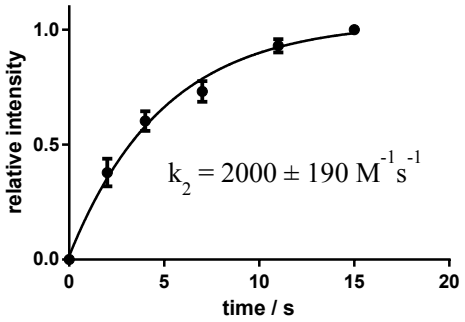

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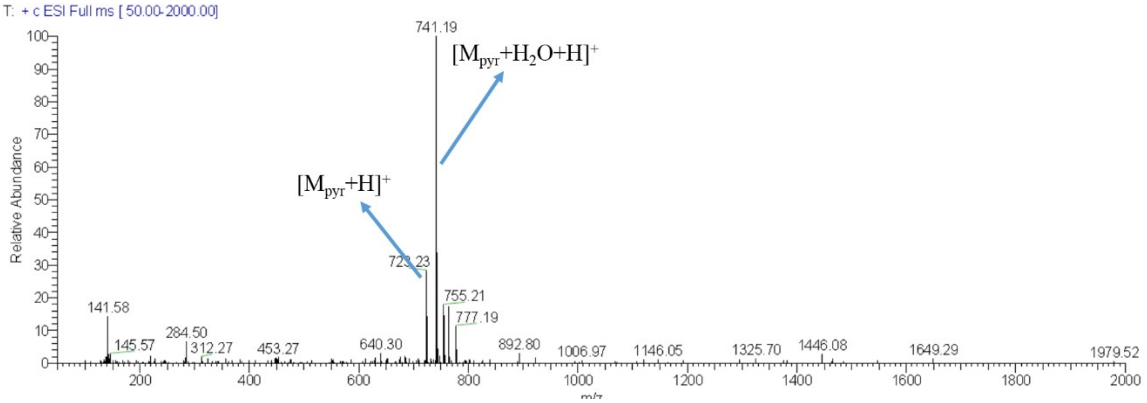

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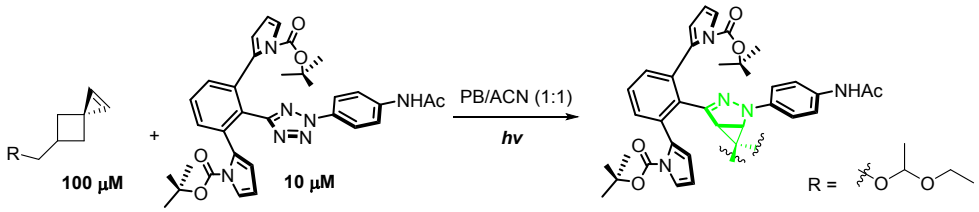

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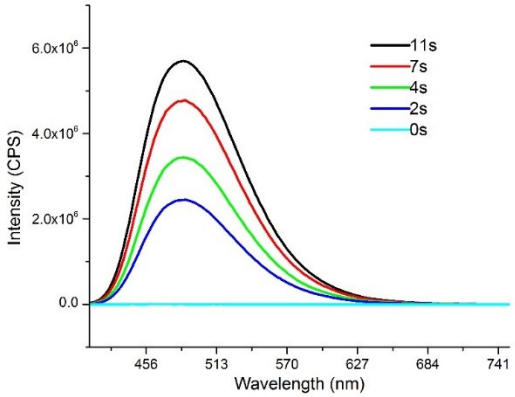

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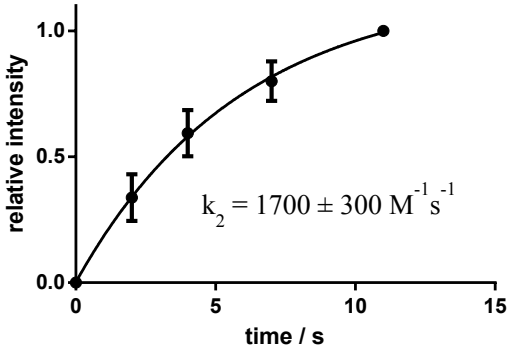

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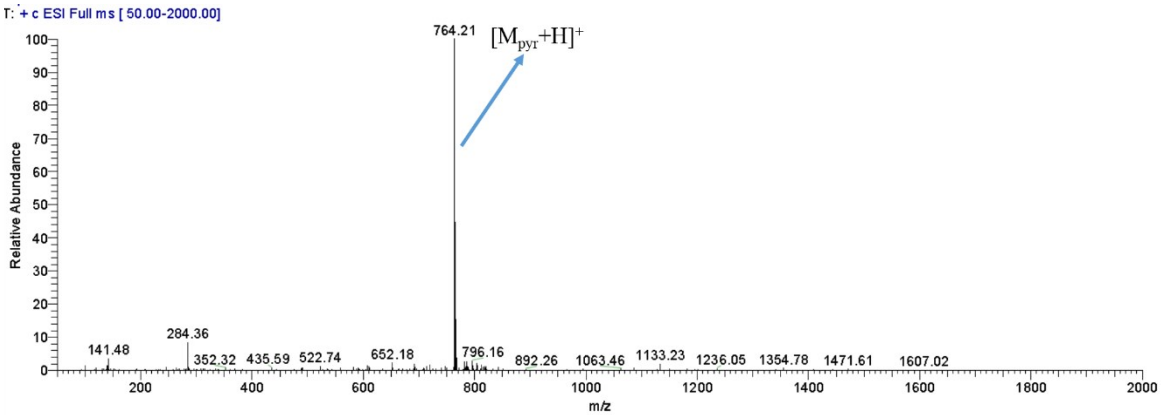

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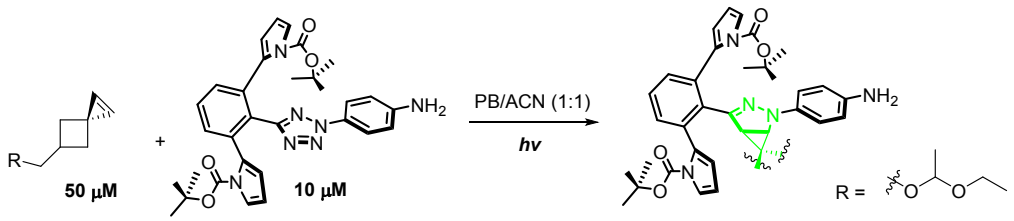

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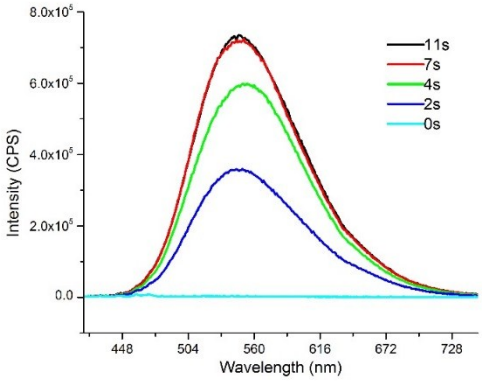

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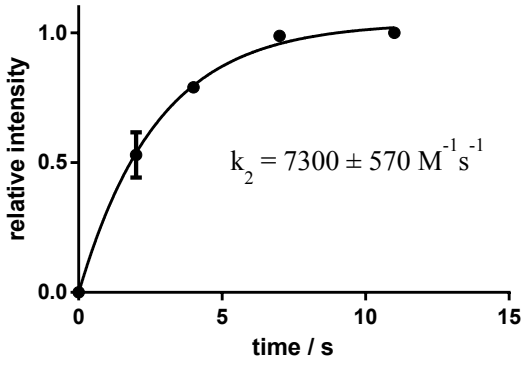

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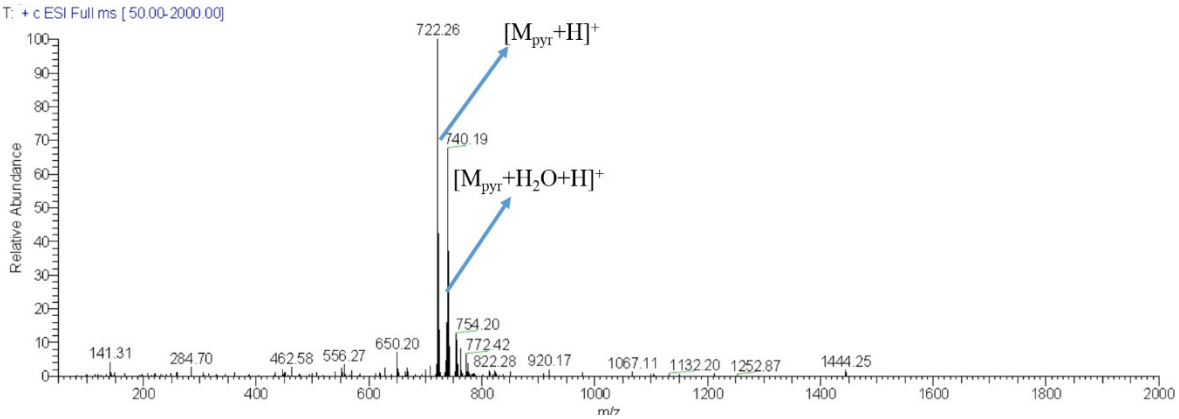

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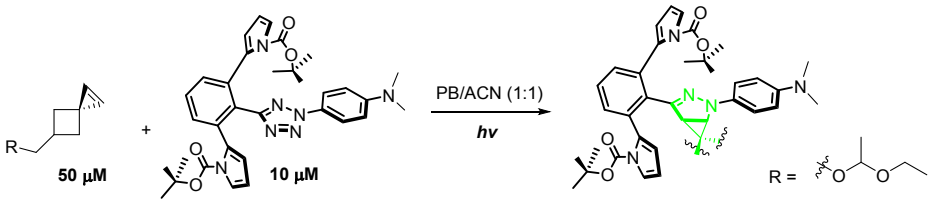

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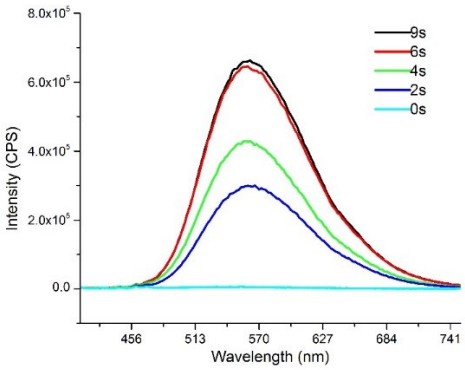

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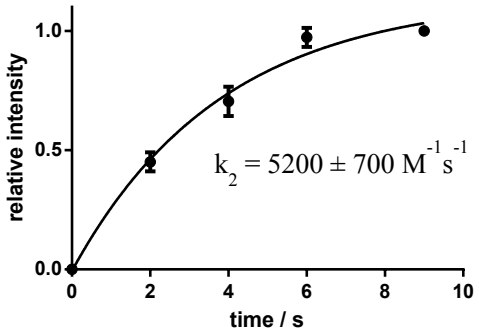

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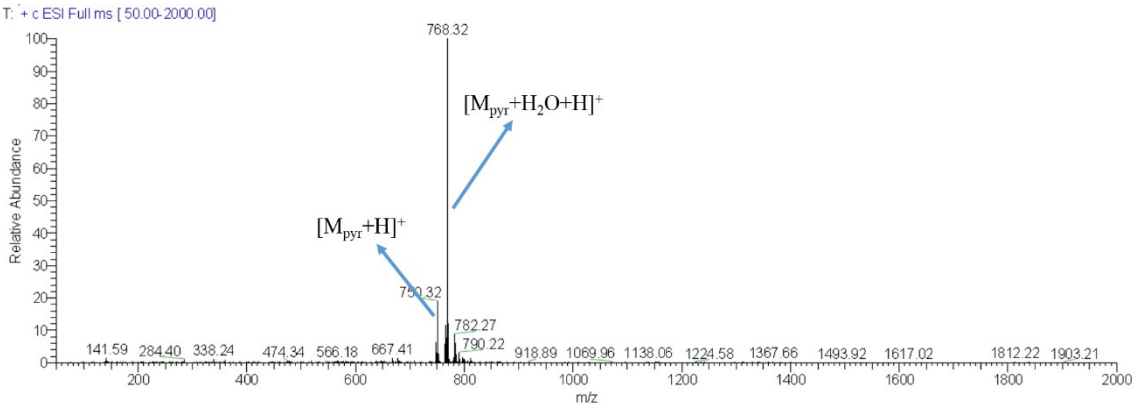

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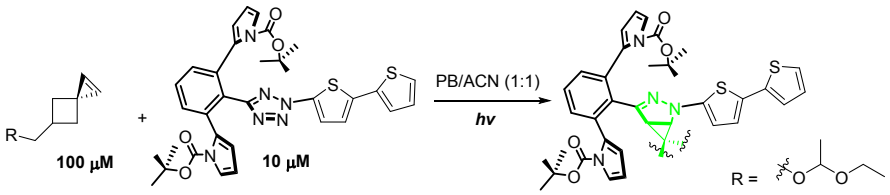

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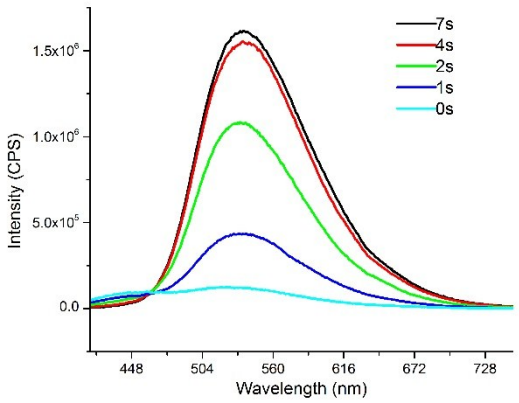

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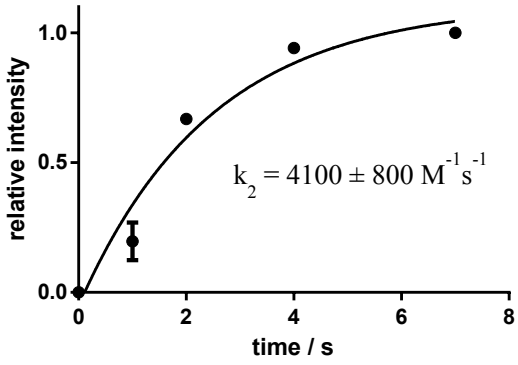

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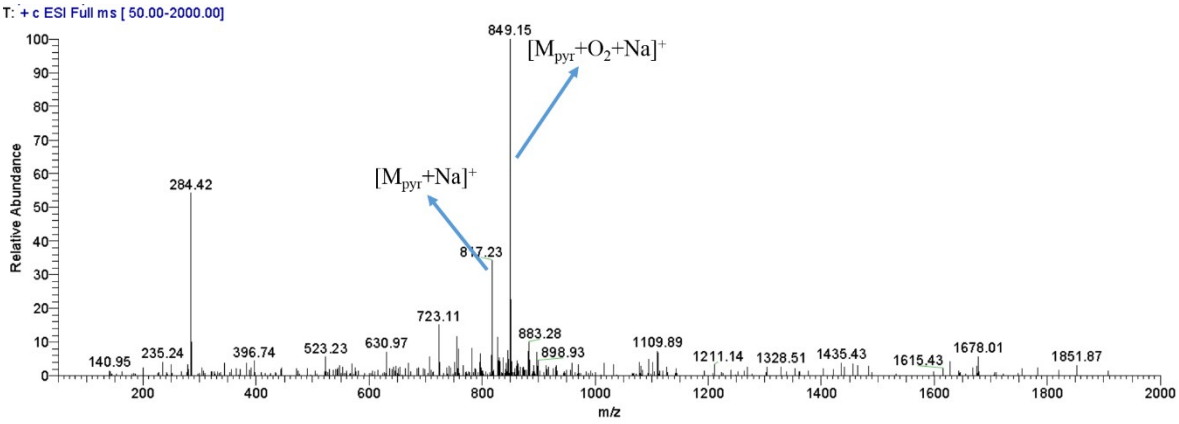

Figure S1. Fluorescence-based kinetics measurement of the cycloaddition between tetrazoles **2-9** (10 μM) and excess amount of Sph in phosphate buffer/acetonitrile (1:1) under 302 nm photoirradiation. (**a**) Reaction scheme. (**b**) Time course of the cycloaddition reaction between the tetrazole and Sph as monitored by a spectrofluorometer. (**c**) Plots of relative fluorescence intensity vs. reaction time. The amounts of pyrazoline adduct were fitted to an exponential rise to maximum equation, $y = (y_0\text{-}a)\, e^{-kt} + a$, to give k. The second-order rate constants, k_2, were calculated using the following equation: $k_2 = k/[\text{dipolarophile}]$. Measurements were repeated three times at each time point to derive the mean and standard deviation. (**d**) LC-MS analysis confirmed the formation of the corresponding pyrazoline cycloadduct.

Tetrazole **2**:

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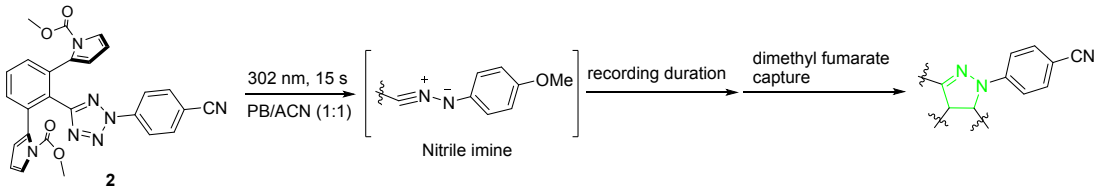

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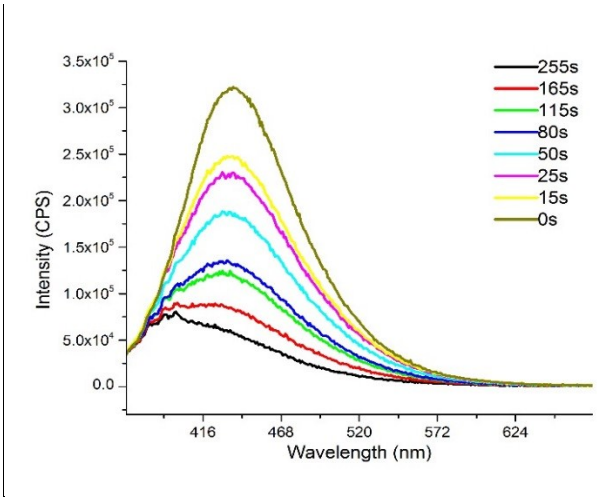

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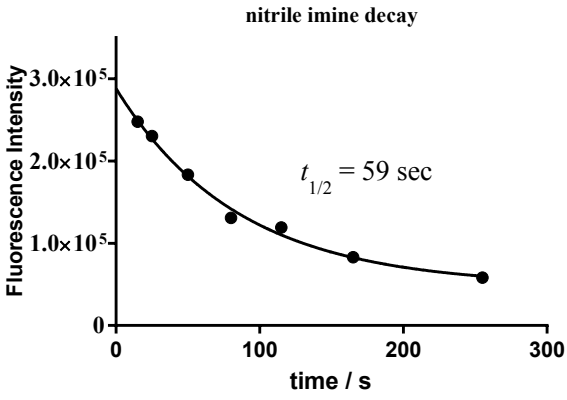

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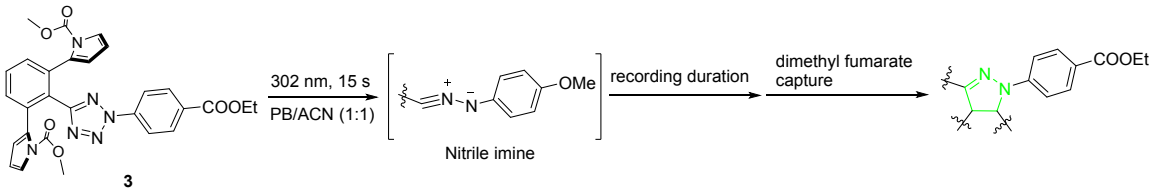

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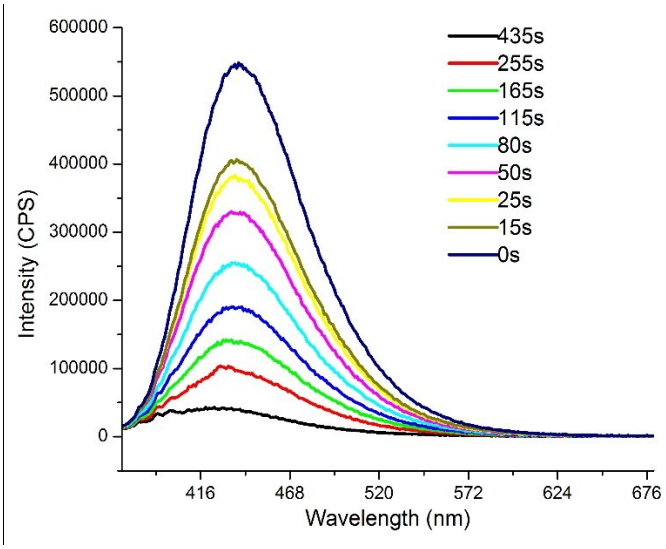

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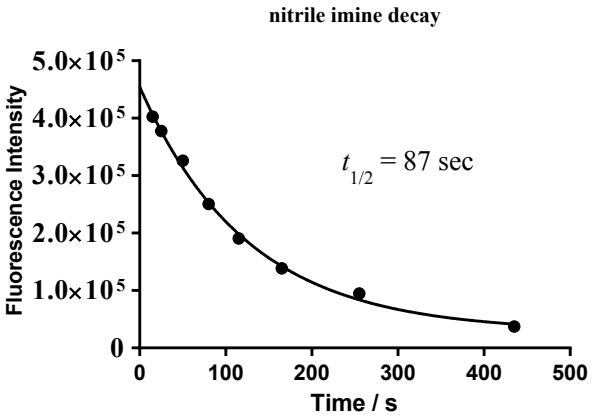

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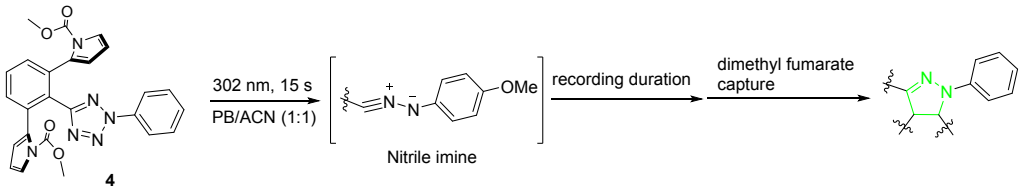

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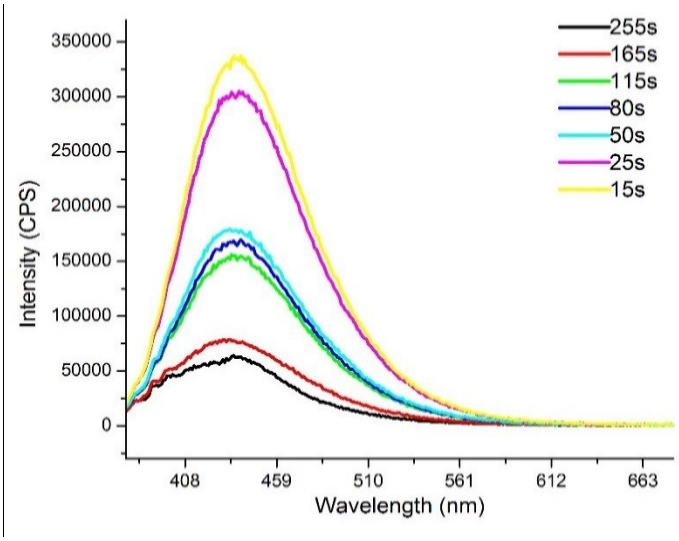

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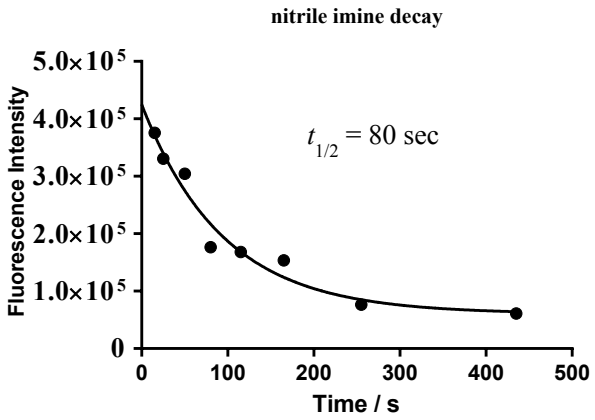

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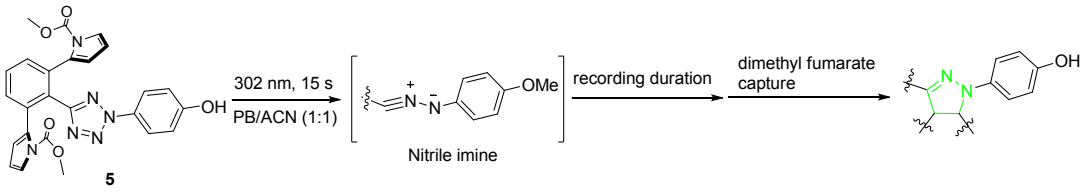

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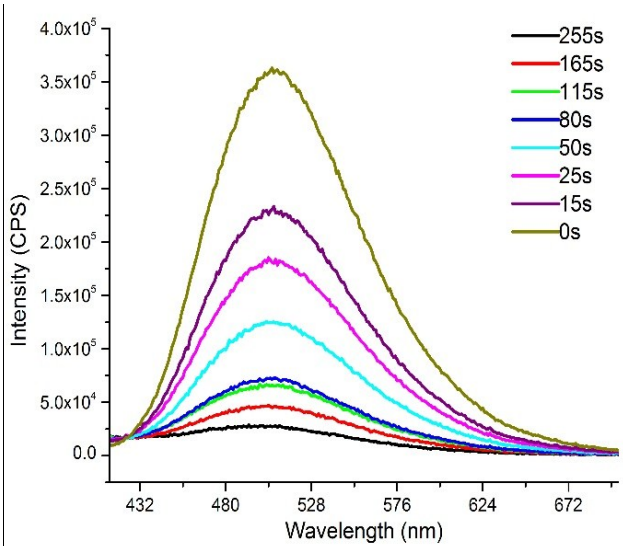

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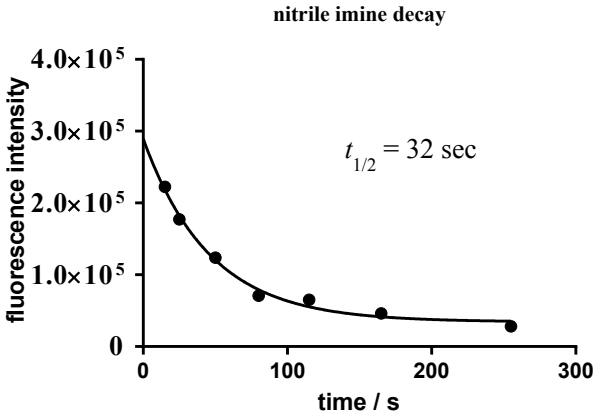

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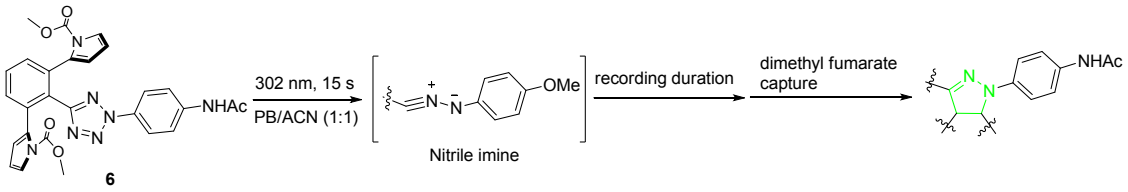

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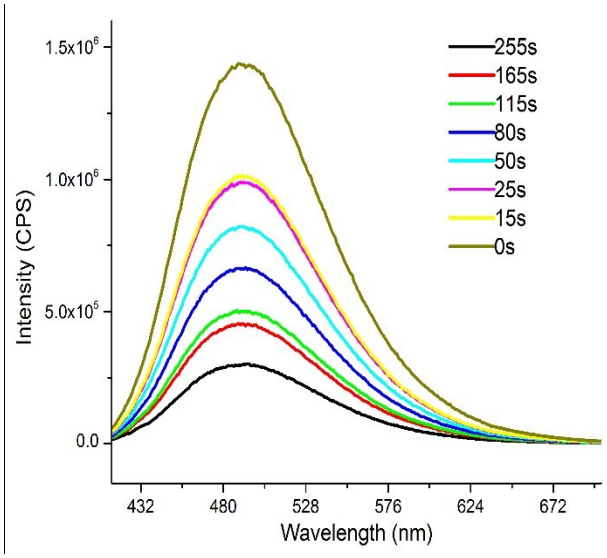

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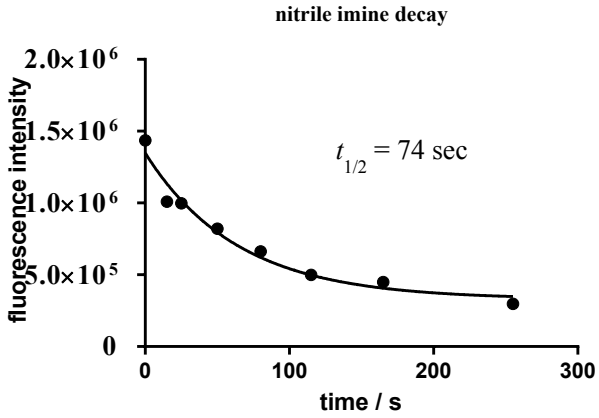

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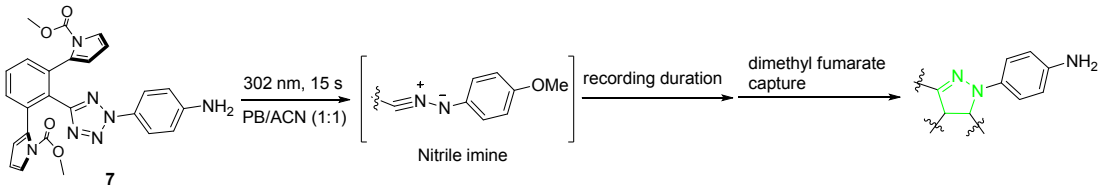

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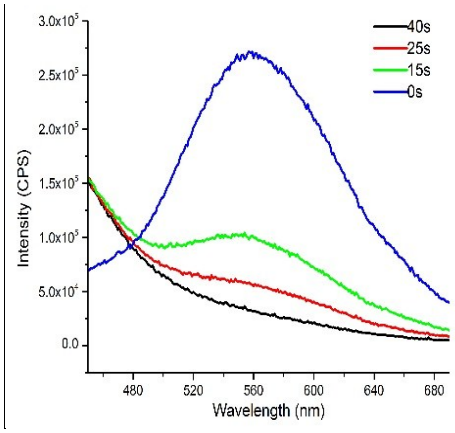

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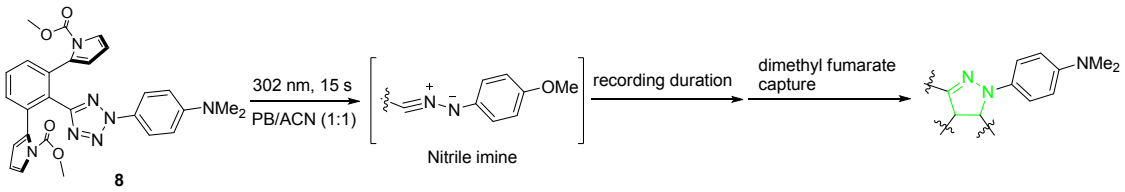

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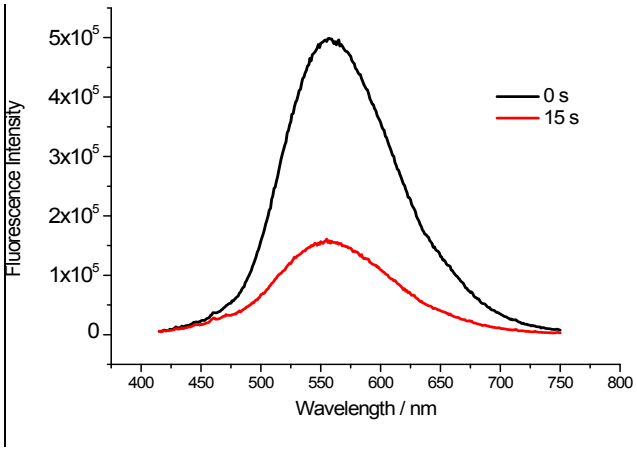

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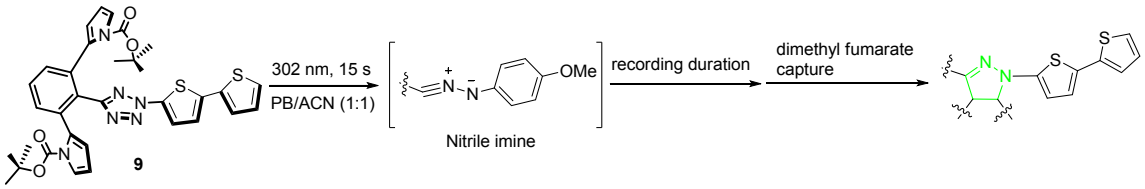

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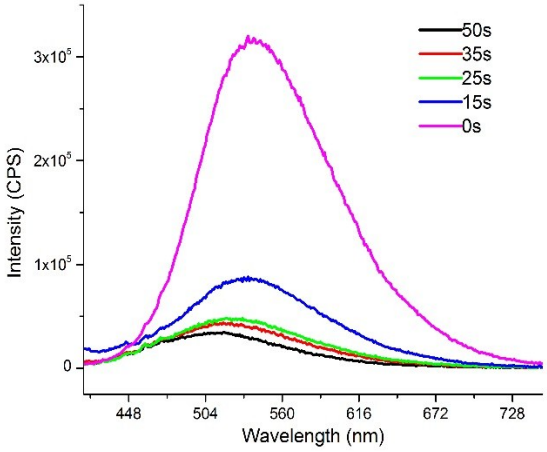
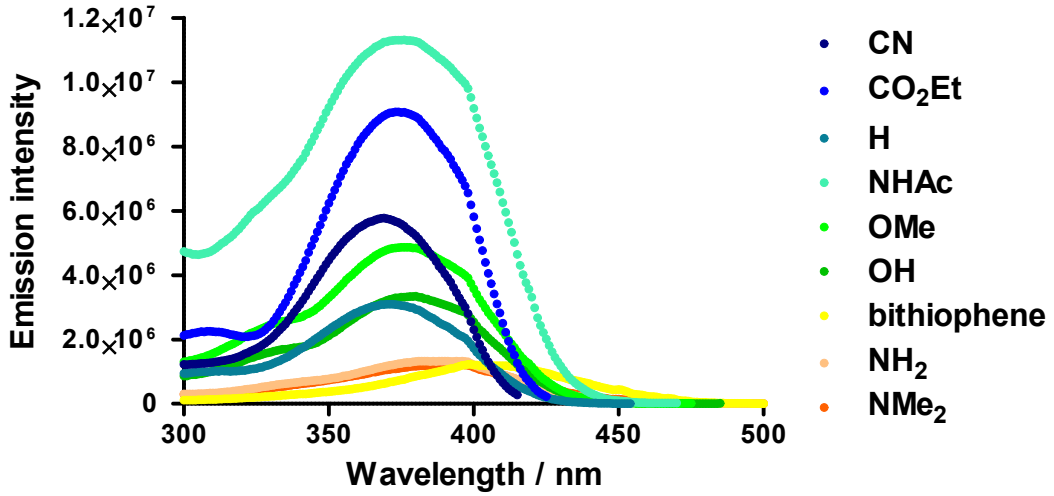


Figure S2. Fluorescence-based determination of half-lives of the nitrile imine in phosphate buffer/acetonitrile (1:1). (**a**) Scheme showing measurement procedure: 10 μM tetrazole was photoirradiated with a handheld 302 nm UV lamp for 15 sec. The resulting solution was left unperturbed for the in situ generated nitrile imine to decay for a period of time before dimethyl fumarate was added with a final concentration of 500 μM to capture the residual nitrile imine to produce the fluorescent pyrazoline product. (**b**) Fluorescence spectra of the pyrazoline products derived from the reaction of dimethyl fumarate with the residual nitrile imine after left unperturbed for various times; λ_{ex} = 350 nm for tetrazoles **2**-**4** and 405 nm for tetrazoles **5**-**9**. The zero-point sample was obtained by incubating dimethyl fumarate with the tetrazole prior to photoirradiation. (**c**) Plot of fluorescence intensity vs. delay time for nitrile imine to obtain the half-life. For nitrile imines **7**-**9**, less than half of the fluorescence was detected after 15 s compared to 0 s, indicating that the half-life of the nitrile imine was shorter than 15 s.

a)



b)

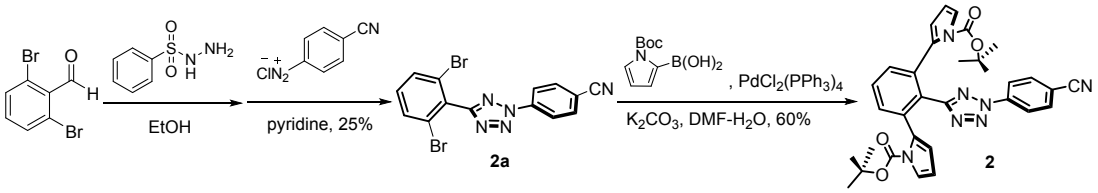
Substituent	CN	CO_2Et	H	NHAc	OMe	OH	Bithio-phene	NH_2	NMe_2
$\lambda_{ex,\,max}$ (nm)	369	374	370	375	377	380	406	387	388
$\lambda_{em,\,max}$ (nm)	430	442	469	486	490	501	536	545	560

Figure S3. (**a**) Excitation spectra of the *in situ* generated pyrazolines in phosphate buffer/acetonitrile (1:1). The emission intensity was monitored at maximum emission of each pyrazoline fluorophore as indicated in the table below. (**b**) Tabulated maximum excitation and emission wavelengths for various pyrazoline fluorophores. Experimental conditions are identical to those in Figure 2.

General Information

Solvents and chemicals were purchased from commercial sources and used directly without further purification. N^{ε}-Boc-lysine (BocK) was purchased from Chem-Impex (Cat. No. 00363). Flash chromatography was performed either manually with SiliCycle P60 silica gel (40-63 μm, 60 Å) or Yamazen AKROS flash system equipped with SiliaSep HP pre-packed columns. ^{1}H NMR spectra were recorded with Inova-300, -400 or -500 MHz spectrometers. Chemical shifts were reported in ppm using TMS or deuterated solvents as internal standards (TMS, 0.00; $CDCl_3$, 7.26; acetone-d_6, 2.05; DMSO-d_6, 2.50). Multiplicity was reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, brs = broad. ^{13}C NMR spectra were recorded at 75.4 MHz or 100 MHz, and chemical shifts were reported in ppm using deuterated solvents as internal standards ($CDCl_3$, 77.0; acetone-d_6, 30.0; DMSO-d_6, 39.5). Fluorescence spectra were recorded on Horiba FluoroMax-4 spectrofluorometer using 1-cm quartz cuvette at 25 ºC. Low resolution mass spectrometry was performed on a Thermo LCQ mass spectrometer. Molecular geometry optimization and orbital energies of nitrile imine were obtained using DFT calculations at B3LYP/6-31G (d) level with Gaussian09 program.[S1]

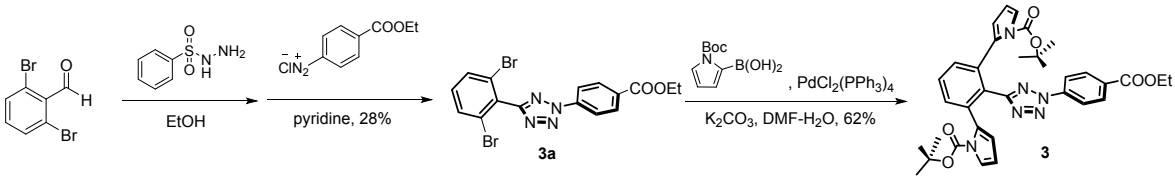
Scheme S1. Synthesis of tetrazole **2**



4-(5-(2,6-Dibromophenyl)-2*H*-tetrazol-2-yl)benzonitrile (**2a**): To a solution of benzenesulfonohydrazide (103 mg, 0.6 mmol) in ethanol was added 2,6-dibromo-benzaldehyde (158 mg, 0.6 mmol), and small amount of ethyl acetate to increase the solubility, and the mixture was stirred at room temperature for 6 hours. The solvent was removed under reduced pressure to give the sulfohydrazone intermediate as a white solid. Preparation of the diazonium salt: to a solution of 4-aminobenzonitrile (705 mg, 6.0 mmol) in ethanol/water (2:1) cooled with ice-water bath was added sequentially 2.0 mL concentrated HCl and 1.0 mL sodium nitrite (454 mg, 6.6 mmol) solution in water, and the mixture was stirred for 10 min. To a solution of the sulfohydrazone intermediate in pyridine (30 mL) cooled with ice-salt bath was added the freshly prepared diazonium salt, and the mixture was stirred while temperature was allowed to gradually rise to room temperature. After about 6 hours, the solvent was removed and DCM (50 mL) was added. The mixture was washed with 1 N HCl before the solvent was removed under reduced pressure. The residue was purified by silica gel flash chromatography using ethyl acetate/hexanes as eluent to give the title compound as a colorless solid (61 mg, 25%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.40 (d, J = 10.0 Hz, 2H), 7.92 (d, J = 10.0 Hz, 2H), 7.71(d, J = 5.0 Hz, 2H), 7.29 (t, J = 5.0 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.13, 139.25, 133.90, 132.78, 132.00, 130.04, 125.20, 120.46, 117.54, 113.68; ESI-MS calcd for $C_{28}H_{15}N_{10}Br_4$ 806.8 [2M+H$^+$], found 806.9.

di-*tert*-Butyl 2,2'-(2-(2-(4-cyanophenyl)-2*H*-tetrazol-5-yl)-1,3-phenylene)bis(1*H*-pyrrole-1-carboxylate) (**2**): To a mixture of tetrazole **2a** (25 mg, 0.06 mmol), (1-(*tert*-butoxycarbonyl)-1*H*-pyrrol-2-yl)boronic acid (46 mg, 0.22 mmol), K_2CO_3 (34 mg, 0.25 mmol) and bis(triphenylphosphine)palladium(II) dichloride (4.3 mg, 0.006 mmol) was added DMF (2 mL) and H_2O (0.3 mL) under argon. The mixture was stirred at 90 °C overnight under argon. The reaction mixture was then cooled down and concentrated under reduced pressure. The residue was purified by silica gel flash chromatography to give the title compound as a white solid (21 mg, 60%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.09 (d, J = 10.0 Hz, 2H), 7.79 (d, J = 10.0 Hz, 2H), 7.53–7.49 (m, 3H), 7.30 (s, 2H), 6.08 (s, 2H), 5.94 (s, 2H), 1.26 (s, 18H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 139.18, 137.64, 135.65, 133.72, 130.84, 128.73, 121.21, 119.84, 117.69, 113.96, 112.88, 110.27, 83.29, 77.40, 76.98, 76.56, 29.68, 27.43; HRMS calcd for $C_{32}H_{32}N_7O_4$ 578.2510 [M+H$^+$], found 578.2515.

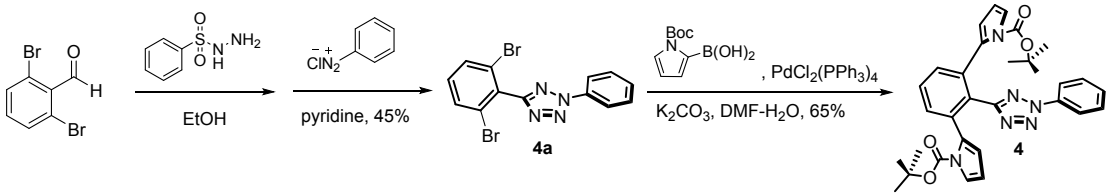
Scheme S2. Synthesis of tetrazole **3**



Ethyl 4-(5-(2,6-dibromophenyl)-2*H*-tetrazol-2-yl)benzoate (**3a**): Tetrazole **3a** was synthesized following the same procedure as tetrazole **2a** with 28% yield: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.32 (d, J = 10.0 Hz, 2H), 8.27 (d, J = 10.0 Hz, 2H), 7.70 (d, J = 8.4 Hz, 2H), 7.27 (t, J = 8.4 Hz, 1H), 4.45 (t, J = 8.4 Hz, 2H), 1.44 (d, J = 8.4 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.21, 163.70, 139.46, 132.59, 131.91, 131.67, 131.19, 125.23, 119.65, 61.50, 14.26; ESI-MS calcd for $C_{16}H_{13}N_4Br_2O_2$ 450.9 [M+H$^+$], found 451.1.

di-*tert*-Butyl 2,2'-(2-(2-(4-(ethoxycarbonyl)phenyl)-2*H*-tetrazol-5-yl)-1,3-phenylene)bis(1*H*-pyrrole-1-carboxylate) (**3**): Tetrazole **3** was synthesized following the same procedure as tetrazole **2** with 62% yield: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.17 (d, J = 8.4 Hz, 2H), 8.03 (d, J = 8.4 Hz, 2H), 7.56 – 7.43 (m, 3H), 7.31 (s, 2H), 6.08 (s, 2H), 5.95 (s, 2H), 4.40 (q, J = 7.0 Hz, 2H), 1.41 (t, J = 7.0 Hz, 3H), 1.26 (s, 18H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.21, 131.04, 130.76, 128.51, 121.19, 119.10, 110.30, 109.93, 83.19, 61.35, 27.42, 14.27; HRMS calcd for $C_{34}H_{37}N_6O_6$ 625.2769 [M+H$^+$], found 625.2796.

Scheme S3. Synthesis of tetrazole **4**

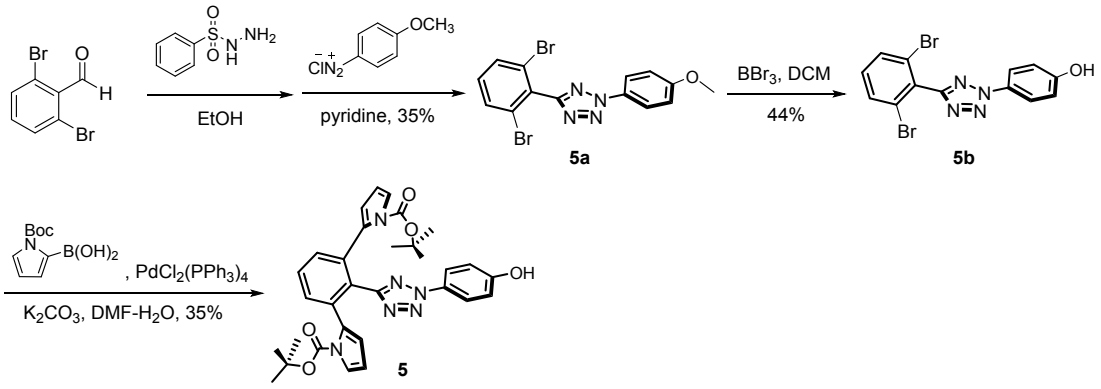


5-(2,6-Dibromophenyl)-2-phenyl-2*H*-tetrazole (**4a**): Tetrazole **4a** was synthesized

using the same procedure as tetrazole **3a** with 45% yield: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.23 (d, J = 8.1 Hz, 2H), 7.69 (d, J = 8.1 Hz, 2H), 7.59 (t, J = 7.7 Hz, 2H), 7.52 (t, J = 7.2 Hz, 1H), 7.25 (t, J = 8.1 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.49, 136.74, 132.45, 131.86, 130.59, 129.87, 129.68, 125.30, 119.92; ESI-MS calcd for $C_{13}H_9N_4Br_2$ 378.9 [M+H$^+$], found 379.2.

di-*tert*-Butyl 2,2'-(2-(2-phenyl-2*H*-tetrazol-5-yl)-1,3-phenylene)bis(1*H*-pyrrole-1-carboxylate) (**3**): Tetrazole **3** was synthesized using the same procedure as tetrazole **2** with 65% yield: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.94 (d, J = 8.1 Hz, 2H), 7.50 – 7.44 (m, 5H), 7.39 (t, J = 7.0 Hz, 1H), 7.31 (s, 2H), 6.08 (t, J = 2.7 Hz, 2H), 5.95 (s, 2H), 1.26 (s, 18H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.58, 149.23, 136.71, 135.81, 131.79, 130.65, 129.42, 129.11, 128.28, 121.14, 119.39, 114.13, 110.15, 83.12, 27.40; HRMS calcd for $C_{31}H_{33}N_6O_4$ 553.2558 [M+H$^+$], found 553.2568.

Scheme S4. Synthesis of tetrazole **5**



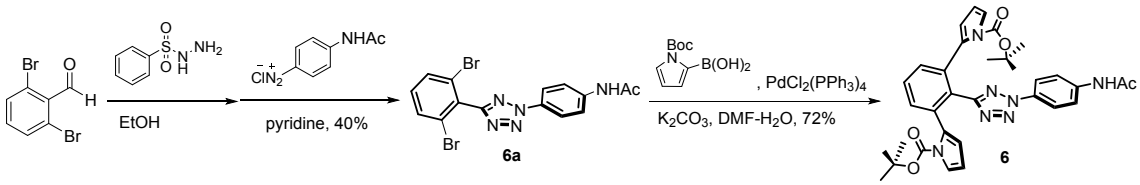
5-(2,6-Dibromophenyl)-2-(4-methoxyphenyl)-2*H*-tetrazole (**5a**): Compound **5a** was reported in our previous study, [S2] and synthesized using the same procedure with 35% yield: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 (d, J = 8.4 Hz, 2H), 7.69 (dd, J = 8.1, 0.7 Hz, 2H), 7.32–7.18 (m, 1H), 7.07 (d, J = 8.4 Hz, 2H), 3.90 (d, J = 0.5 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.28, 160.68, 132.39, 131.87, 130.74, 130.30, 125.36, 121.50, 114.71, 55.67; ESI-MS calcd for $C_{14}H_{11}N_4Br_2O$ 408.9 [M+H$^+$], found 409.0.

4-(5-(2,6-Dibromophenyl)-2*H*-tetrazol-2-yl)phenol (**5b**): To a solution of tetrazole **5a** (100 mg, 0.24 mmol) in DCM (15 mL) cooled with ice-water bath was slowly added boron tribromide (0.23 mL, 2.4 mmol), and the mixture was stirred at room temperature overnight. After cooling the reaction mixture with ice-water bath, 1 mL methanol was slowly added and the solution was concentrated. The residue was purified by silica gel flash chromatography to give the titled compound as a white solid (42 mg, 44%): ^{1}H NMR (400 MHz, acetone-d_6) δ 8.11 – 7.99 (m, 2H), 7.88 (d, J = 8.1 Hz, 2H), 7.50 (t, J = 8.1 Hz, 1H), 7.18 – 7.01 (m, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 163.27, 159.11, 133.34, 132.20, 130.84, 129.33, 124.87, 121.64, 116.36; ESI-MS calcd for $C_{13}H_9N_4Br_2O$ 394.9 [M+H$^+$], found 395.1.

di-*tert*-Butyl 2,2'-(2-(2-(4-hydroxyphenyl)-2*H*-tetrazol-5-yl)-1,3-phenylene)bis

(1*H*-pyrrole-1-carboxylate) (**5**): Tetrazole **5** was synthesized using the same procedure as tetrazole **2** with 35% yield: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.87 (d, J = 8.4 Hz, 2H), 7.54 – 7.43 (m, 3H), 7.32 (s, 2H), 6.95 (d, J = 8.9 Hz, 2H), 6.06 (t, J = 3.0 Hz, 2H), 5.89 (s, 2H), 1.26 (s, 18H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.79, 157.07, 149.71, 135.77, 131.88, 130.83, 130.02, 128.23, 121.19, 121.02, 116.10, 110.35, 83.49, 27.44; HRMS calcd for $C_{31}H_{33}N_6O_5$ 569.2507 [M+H$^+$], found 569.2523.

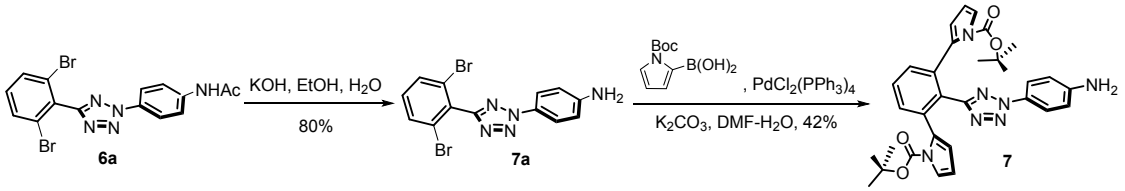
Scheme S5. Synthesis of tetrazole **6**



***N*-(4-(5-(2,6-Dibromophenyl)-2*H*-tetrazol-2-yl)phenyl)acetamide** (**6a**): Tetrazole **6a** was synthesized using the same procedure as tetrazole **2a** with 40% yield: ^{1}H NMR (500 MHz, acetone-d_6) δ 9.55 (s, 1H), 8.15 (d, J = 8.8 Hz, 2H), 7.97 (d, J = 8.8 Hz, 2H), 7.88 (d, J = 8.1 Hz, 2H), 7.50 (t, J = 8.2 Hz, 1H), 2.15 (s, 3H); ^{13}C NMR (75 MHz, acetone-d_6) δ 168.50, 163.42, 141.41, 133.41, 132.22, 131.64, 130.70, 124.85, 120.49, 119.80, 119.72, 29.74, 23.49; ESI-MS calcd for $C_{15}H_{12}N_5Br_2O$ 435.9 [M+H$^+$], found 436.1.

di-*tert*-Butyl 2,2'-(2-(2-(4-acetamidophenyl)-2*H*-tetrazol-5-yl)-1,3-phenylene)bis (1*H*-pyrrole-1-carboxylate) (**6**): Tetrazole **6** was synthesized using the same procedure as tetrazole **2** with 72% yield: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 (s, 1H), 7.92 (d, J = 8.7 Hz, 2H), 7.70 (d, J = 8.6 Hz, 2H), 7.53 – 7.47 (m, 3H), 7.31 (s, 2H), 6.06 (s, 2H), 5.91 (s, 2H), 2.21 (s, 3H), 1.26 (s, 18H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.82, 149.50, 139.37, 135.76, 132.28, 132.04, 131.91, 130.78, 128.65, 128.49, 128.28, 120.99, 120.16, 120.01, 110.30, 83.30, 77.41, 76.98, 76.56, 27.41, 24.48; HRMS calcd for $C_{33}H_{35}N_7NaO_5$ 632.2592 [M+Na$^+$], found 632.2585.

Scheme S6. Synthesis of tetrazole **7**

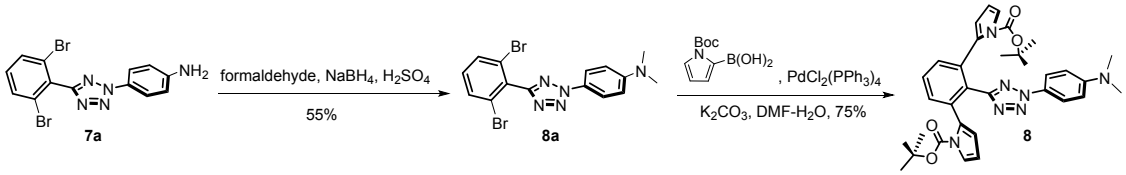


4-(5-(2,6-Dibromophenyl)-2*H*-tetrazol-2-yl)aniline (**7a**): To a solution of tetrazole **6a** (100 mg, 0.22 mmol) in ethanol (15 mL) was added KOH (64 mg, 1.14 mmol) solution in water (2 mL), and the mixture was stirred as 100 °C overnight. The solvent was removed and 5 mL water and DCM were used. The organic layer was separated and dried over Na_2SO_4. The organic solvent was removed under reduced pressure, and the residue was purified by silica gel flash chromatography using ethyl acetate/hexanes as eluent to give the title compound as a colorless solid (72 mg, 80%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 (d, J = 8.8 Hz, 2H), 7.66 (d, J = 8.1 Hz, 2H), 7.22 (t, J = 8.1 Hz,

1H), 6.77 (d, J = 8.8 Hz, 2H), 4.08 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.90, 148.19, 148.13, 132.32, 131.77, 130.71, 128.10, 125.26, 121.37, 114.87, 77.33, 77.01, 76.69; ESI-MS calcd for $C_{13}H_{10}N_5Br_2$ 393.9 [M+H$^+$], found 394.1.

di-*tert*-butyl 2,2'-(2-(2-(4-aminophenyl)-2*H*-tetrazol-5-yl)-1,3-phenylene)bis(1*H*-pyrrole-1-carboxylate) (**7**): Tetrazole **7** was synthesized using the same procedure as tetrazole **2** with 42% yield: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 (d, J = 8.8 Hz, 2H), 7.52 – 7.36 (m, 3H), 7.30 (s, 2H), 6.69 (d, J = 8.9 Hz, 2H), 6.07 (s, 2H), 5.93 (s, 2H), 3.87 (s, 2H), 1.26 (s, 18H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.04, 149.30, 147.37, 135.78, 131.99, 130.63, 128.59, 128.09, 121.09, 120.94, 114.91, 113.90, 110.13, 83.10, 27.43; HRMS calcd for $C_{31}H_{34}N_7O_4$ 568.2667 [M+H$^+$], found 568.2681.

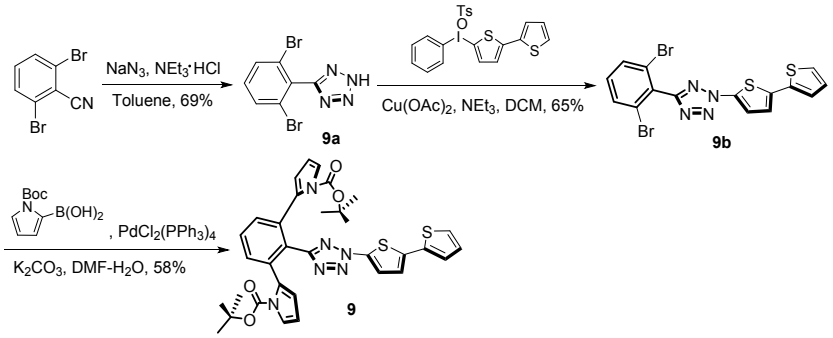
Scheme S7. Synthesis of tetrazole **8**



4-(5-(2,6-Dibromophenyl)-2*H*-tetrazol-2-yl)-N,N-dimethylaniline (**8a**): To a stirred mixture of 3 N H_2SO_4 and 40% aqueous formaldehyde (0.13 mL, 1.52 mmol) at 0 °C was added a slurry of tetrazole **7a** (60 mg, 0.15 mmol), $NaBH_4$ (69 mg, 1.82 mmol) in THF. The reaction was allowed to warm up to room temperature until TLC showed no starting material remaining. The pH of the reaction mixture was adjusted to 10 using aqueous NaOH. The mixture was extracted with ethyl acetate and the organic layer was separated and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified by silica gel flash chromatography to give the title compound as a colorless solid (35 mg, 55%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 (d, J = 9.2 Hz, 5H), 7.68 (d, J = 8.1 Hz, 5H), 7.23 (t, J = 8.1 Hz, 3H), 6.79 (d, J = 9.2 Hz, 6H), 3.05 (s, 18H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.06, 151.18, 132.25, 131.82, 131.03, 126.49, 125.44, 121.14, 111.88, 40.35; ESI-MS calcd for $C_{15}H_{14}N_5Br_2$ 422.0 [M+H$^+$], found 422.1.

di-*tert*-Butyl 2,2'-(2-(2-(4-(dimethylamino)phenyl)-2*H*-tetrazol-5-yl)-1,3-phenylene)bis(1*H*-pyrrole-1-carboxylate) (**8**): Tetrazole **8** was synthesized using the same procedure as tetrazole **2** with 75% yield: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, J = 9.1 Hz, 2H), 7.54 – 7.40 (m, 3H), 7.31 (s, 2H), 6.71 (d, J = 9.2 Hz, 2H), 6.07 (t, J = 3.2 Hz, 2H), 5.92 (s, 2H), 3.00 (s, 6H), 1.26 (s, 18H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 150.69, 135.73, 130.61, 127.98, 126.71, 121.07, 120.57, 111.87, 110.09, 83.04, 77.31, 76.99, 76.68, 40.34, 27.43; HRMS calcd for $C_{33}H_{38}N_7O_4$ 596.2980 [M+H$^+$], found 596.2994.

Scheme S8. Synthesis of tetrazole **9**



5-(2,6-Dibromophenyl)-2*H*-tetrazole (**9a**): A mixture of 2,6-dibromobenzonitrile (200 mg, 0.77 mmol), NaN_3 (149 mg, 2.3 mmol), and triethyl amine hydrochloride salt (315 mg, 2.3 mmol) in toluene was heated to 110 °C for 20 hours. After cooling to room temperature, the product was extracted with water, then concentrated HCl was added to the water phase to precipitate the tetrazole. After filtration, tetrazole **9a** was collected and dried, which was used directly in the next step without further purification (160 mg, 69%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.90 (d, J = 8.1 Hz, 2H), 7.51 (t, J = 8.1 Hz, 1H); ESI-MS calcd for $C_7H_5N_4Br_4$ 302.9 [M+H$^+$], found 303.0.

2-((2,2'-Bithiophen)-5-yl)-5-(2,6-dibromophenyl)-2*H*-tetrazole (**9b**): To a mixture of tetrazole **9a** (50 mg, 0.164 mmol), iodonium salt (266 mg, 0.493 mmol) in DCM (10 mL) was added $Cu(OAc)_2$ (30 mg, 0.164 mmol) and triethyl amine (92 μL, 0.658 mmol), and the mixture was stirred overnight. The solvent was removed under reduced pressure and the residue was purified by silica gel flash chromatography using ethyl acetate /hexanes as eluent to give the title compound as a colorless solid (50 mg, 65%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.69 (d, J = 8.1 Hz, 2H), 7.65 (d, J = 4.0 Hz, 1H), 7.32 (dd, J = 5.1, 0.9 Hz, 1H), 7.28 – 7.23 (m, 2H), 7.14 (d, J = 4.0 Hz, 1H), 7.07 (dd, J = 5.0, 3.7 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.36, 136.35, 135.60, 135.42, 132.61, 131.92, 130.17, 128.14, 125.89, 125.35, 124.95, 122.60, 119.98; ESI-MS calcd for $C_{15}H_{19}N_4Br_2S_2$ 466.9 [M+H$^+$], found 470.0.

di-*tert*-Butyl 2,2'-(2-(2-([2,2'-bithiophen]-5-yl)-2*H*-tetrazol-5-yl)-1,3-phenylene) bis(1*H*-pyrrole-1-carboxylate) (**9**): Tetrazole **9** was synthesized using the same procedure as tetrazole **2** with 58% yield: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.51-7.44 (m, 3H), 7.36 (s, 1H), 7.32 (s, 2H), 7.28-7.25 (m, 1H), 7.19 (d, J = 2.6 Hz, 1H), 7.03 (dd, J = 9.4, 3.8 Hz, 2H), 6.09 (s, 2H), 5.94 (s, 2H), 1.28 (s, 18H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 149.17, 135.85, 135.35, 130.70, 129.39, 128.45, 128.01, 125.54, 124.66, 123.22, 122.60, 121.26, 118.96, 116.19, 110.77, 110.16, 83.16, 27.46; HRMS calcd for $C_{33}H_{32}N_6NaO_4S_2$ 663.1819 [M+Na$^+$], found 663.1841.

Expression and purification of unnatural amino acid-containing sfGFP mutants. BL21(DE3) cells were transformed with the pEvol-PylT-mmPylRS and pET-sfGFP-T65G/Y66G/Q204TAG plasmids. The cells were recovered in 1 mL SOC medium following transformation and incubated at 37 ºC for 1 hour before plating on LB agar plate containing chloramphenicol (Cam) (34 μg/mL) and ampicillin (Amp) (100

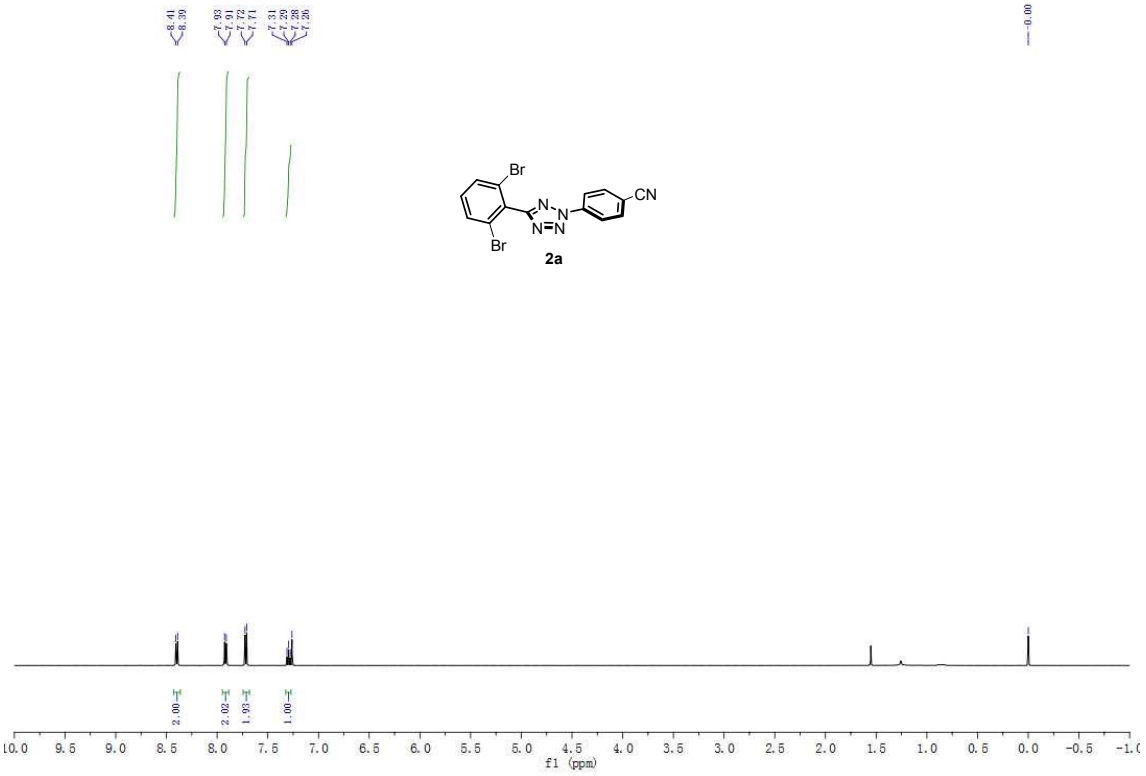
μg/mL). A 5-mL overnight culture from a single colony was used to inoculate 25 mL LB medium supplemented with Cam and Amp. Cells were grown at 37 ºC in a shaker-incubator (250 rpm), and the protein expression was induced by adding 1 mM IPTG, 0.2% arabinose, and 1 mM SphK for sfGFP-Q204SphK or 1 mM BocK for sfGFP-Q204BocK when OD_{600} reached 0.6. After 6-hour induction, cells were harvested, resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), and sonicated in ice/water bath ten times (10 sec each with 20 sec interval). The lysate was centrifuged (30 min, 4 ºC). The supernatant was incubated with 0.1 mL Ni-NTA resin (Thermo HisPur™) (2 hours, 25 ºC). The slurry was then loaded to a column and the protein-bound resin was washed twice with 3 mL washing buffer (50 mM Na_2HPO_4, 300 mM NaCl, 50 mM imidazole, pH 8.0). The protein was eluted off the resin using the elution buffer (50 mM Na_2HPO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The eluted fractions were collected, concentrated, and buffer-exchanged to DPBS containing 100 μM TCEP using 10 kD MWCO spin column column (Thermo Scientific). The protein identity was verified by LC/ESI-MS and the concentration was determined by BCA essay. For SDS-PAGE analysis, 10 μM protein in PBS/ACN (1:1) mixed solvent was added 200 μM tetrazole and the mixture was irradiated with a handheld 302-nm UV lamp for 30 sec. The fluorescence was recorded using Horiba FluoroMax-4 spectrofluorometer. The loading buffer was then added and the sample was heated to 100 ºC for 10 min before being loaded to the gel. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on an XCell SureLock Mini-Cell apparatus using the precast ExpressPlus 4-12% PAGE gel (GenScript). BenchMark Prestained Protein Ladder was applied to one lane of the gel for estimation of apparent molecular weight.

Reference:

[S1] Gaussian 09, Revision A.02, M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H. P. Hratchian, A. F. Izmaylov, J. Bloino, G. Zheng, J. L. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J. A. Montgomery, Jr., J. E. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, K. N. Kudin, V. N. Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J. M. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, R. L. Martin, K. Morokuma, V. G. Zakrzewski, G. A. Voth, P. Salvador, J. J. Dannenberg, S. Dapprich, A. D. Daniels, O. Farkas, J. B. Foresman, J. V. Ortiz, J. Cioslowski, D. J. Fox, Gaussian, Inc., Wallingford CT, 2009.

[S2] P. An, T. Lewandowski, T. G. Erbay, P. Liu, Q. Lin, *J. Am. Chem. Soc.* **2018**, *140*, 4860.

NMR spectra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N
N
CN
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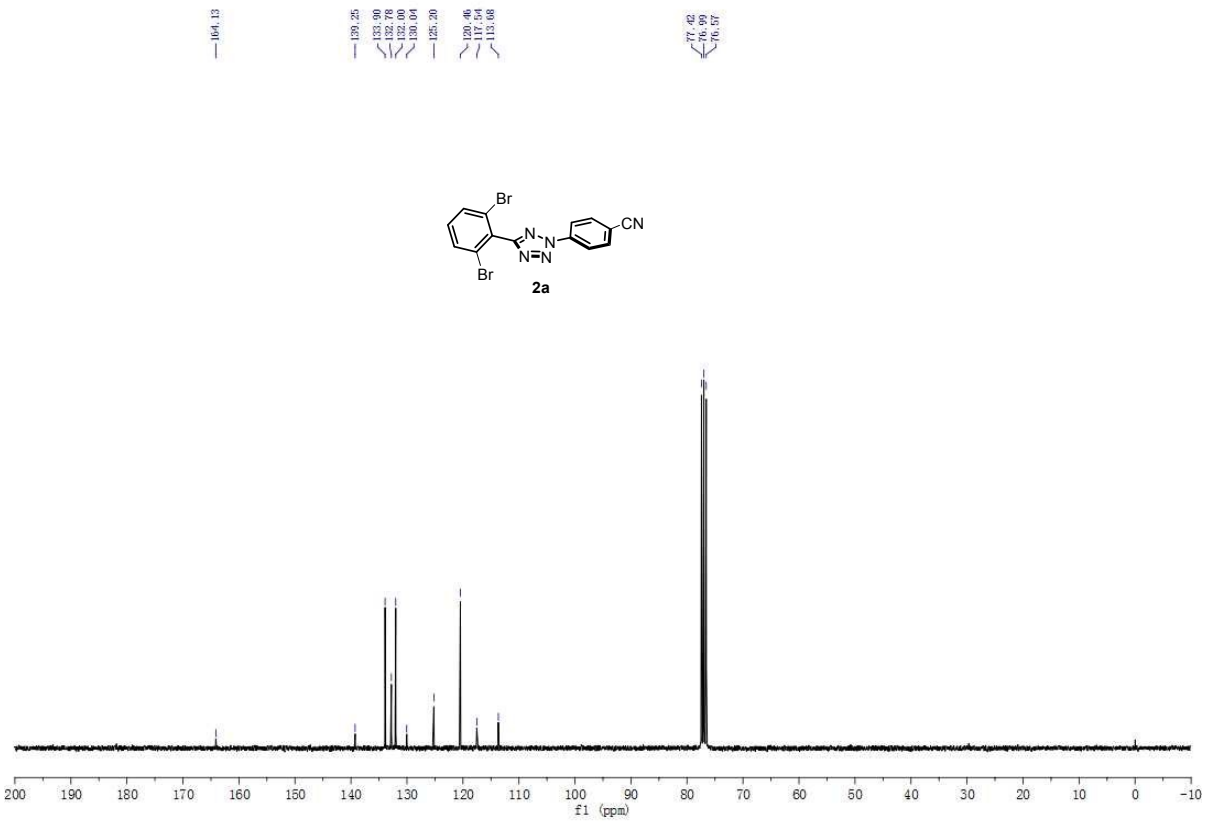
f1 (ppm)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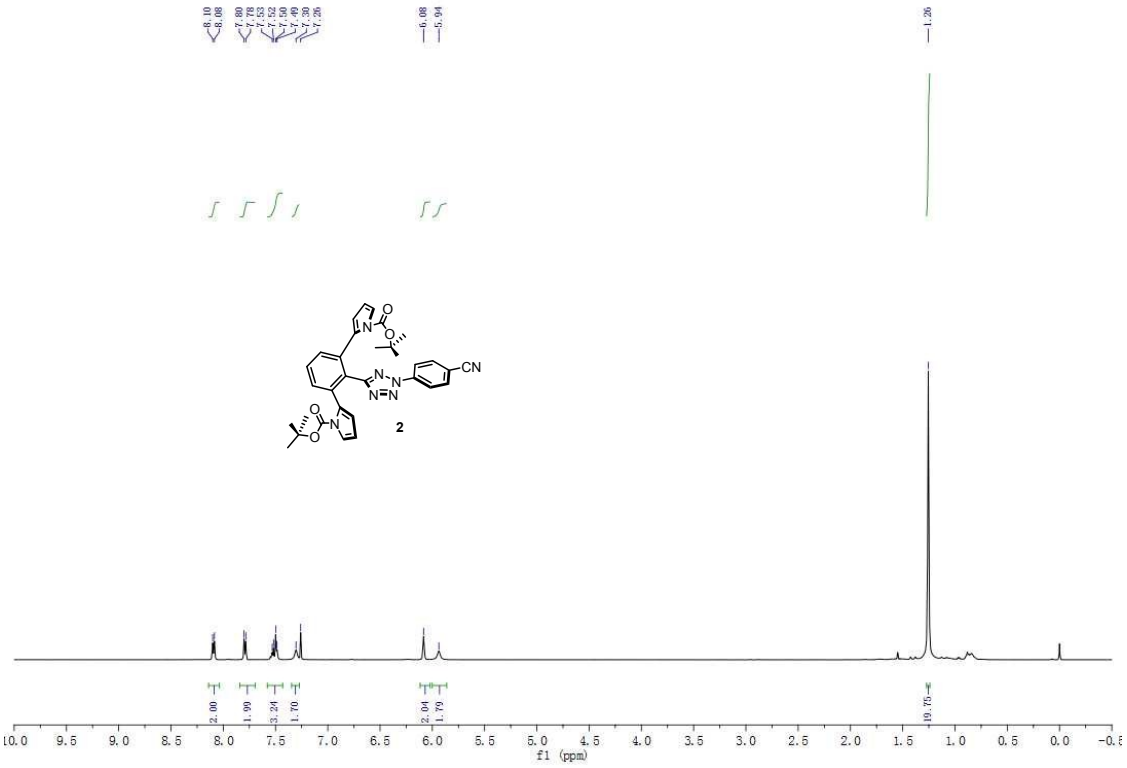
f1 (ppm)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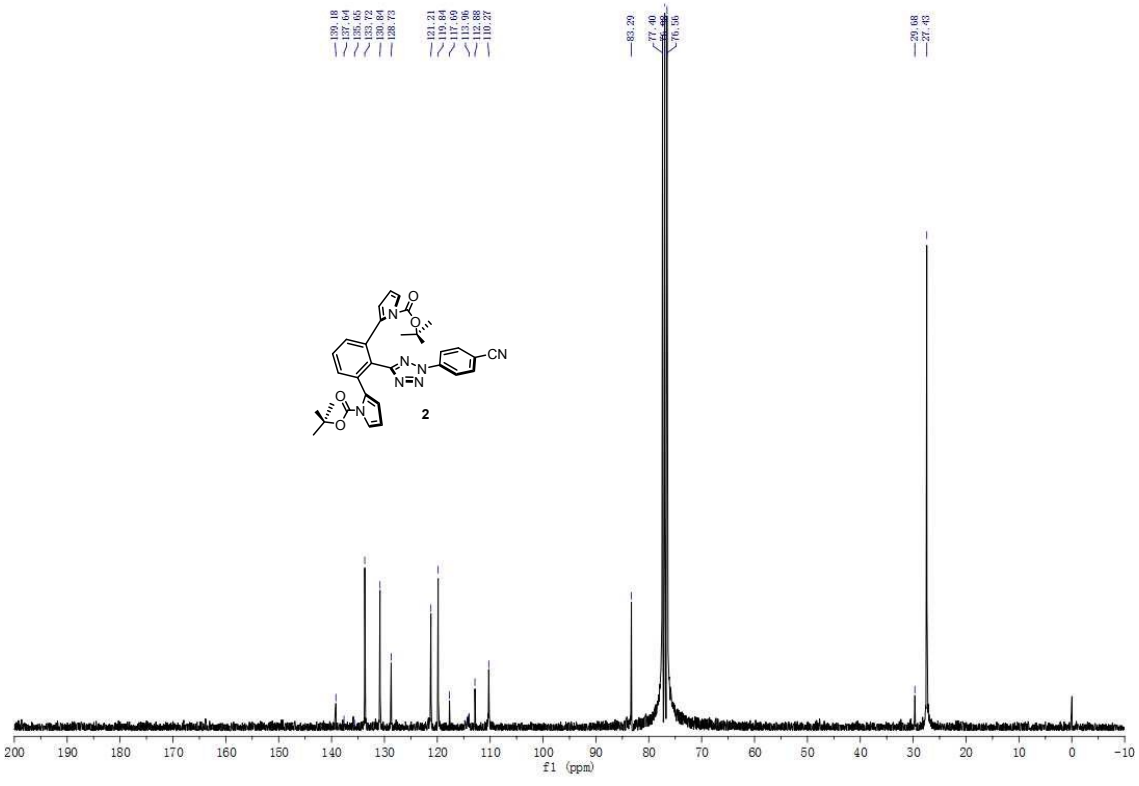
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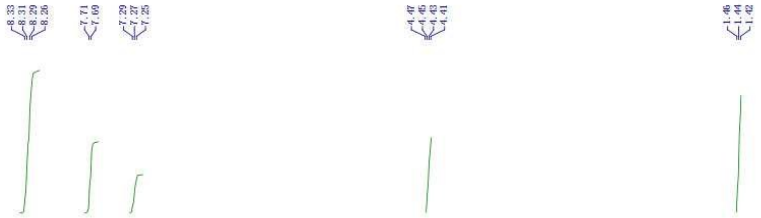
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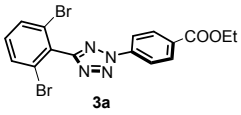
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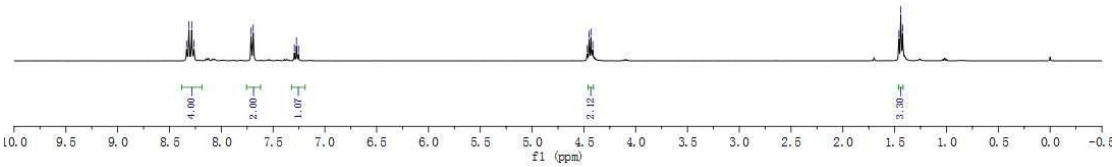
N

3a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3.0
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2.0
1.5
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f1 (ppm)


165.21
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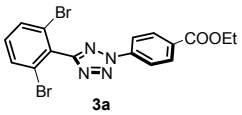
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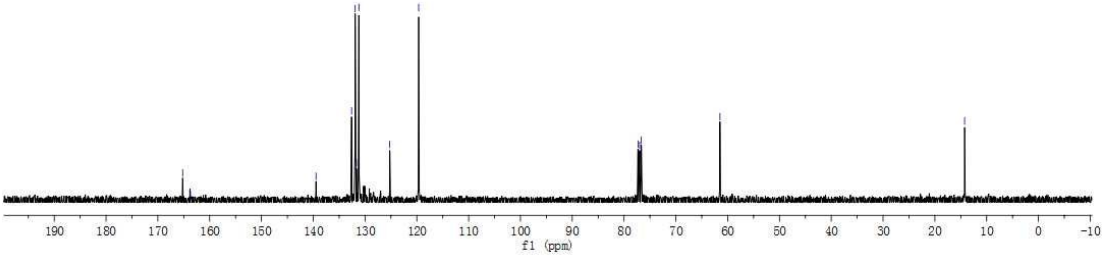
N

3a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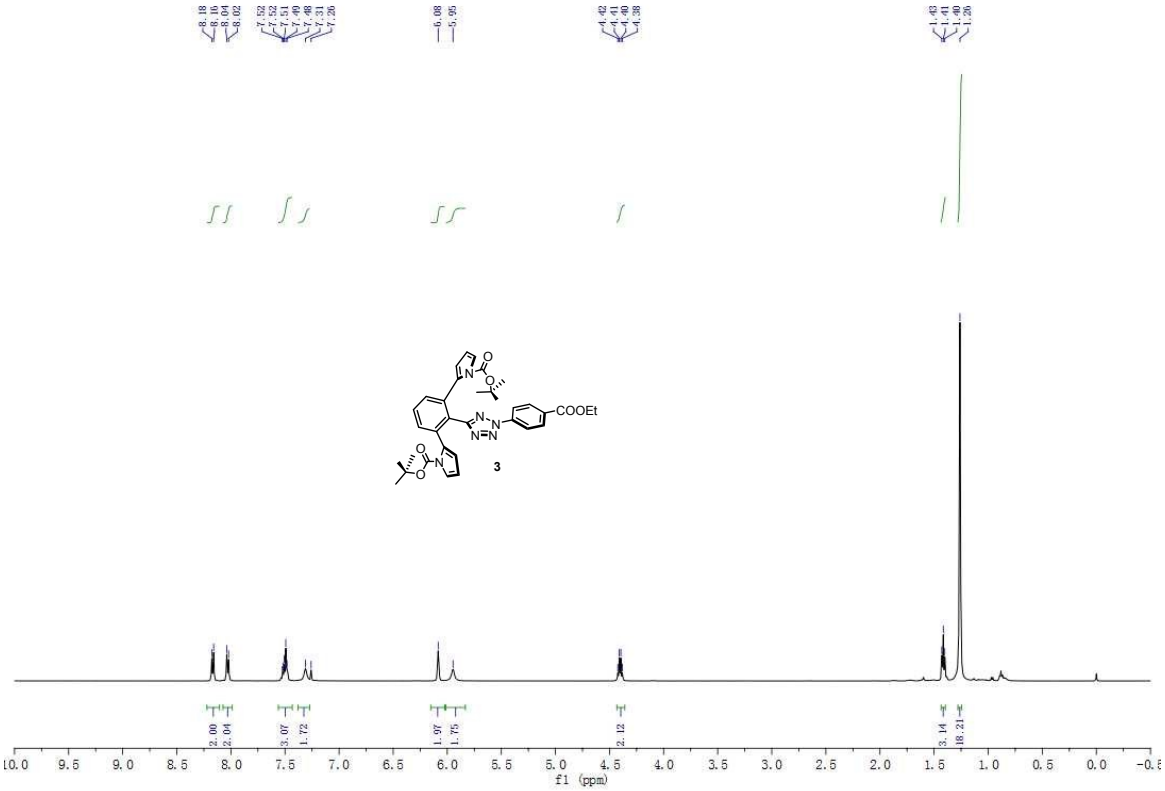
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4.41
4.40
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1.43
1.41
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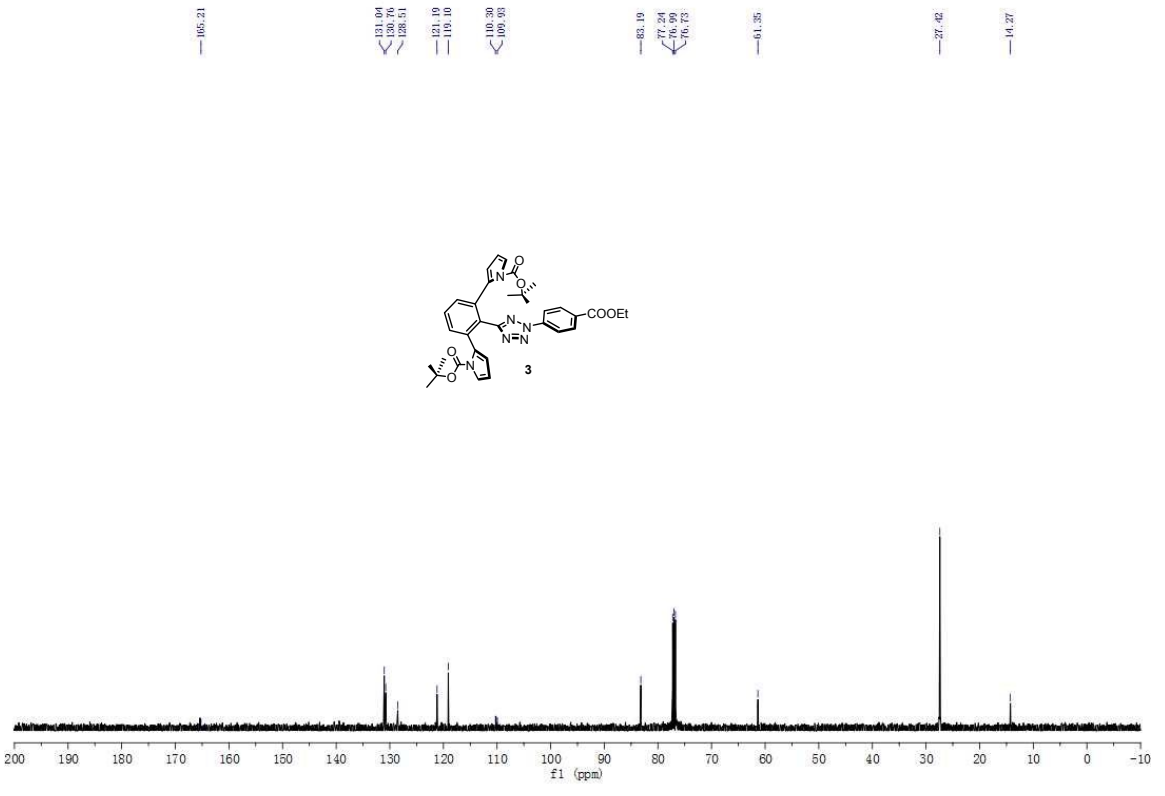
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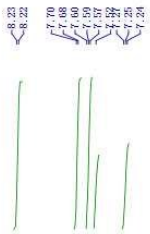
f1 (ppm)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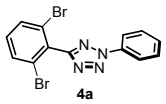
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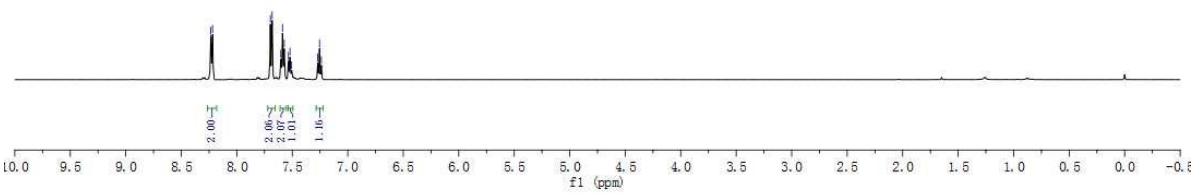
4a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f1 (ppm)


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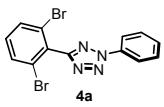
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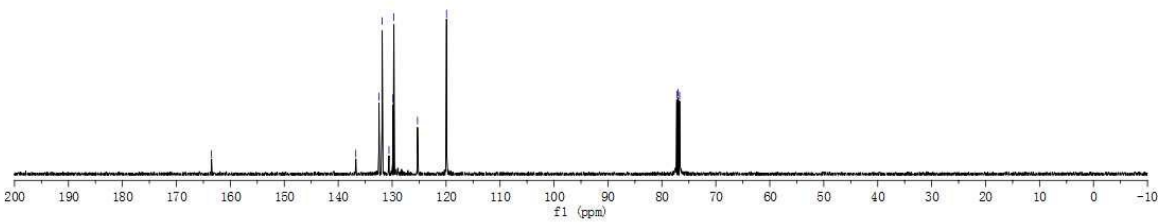
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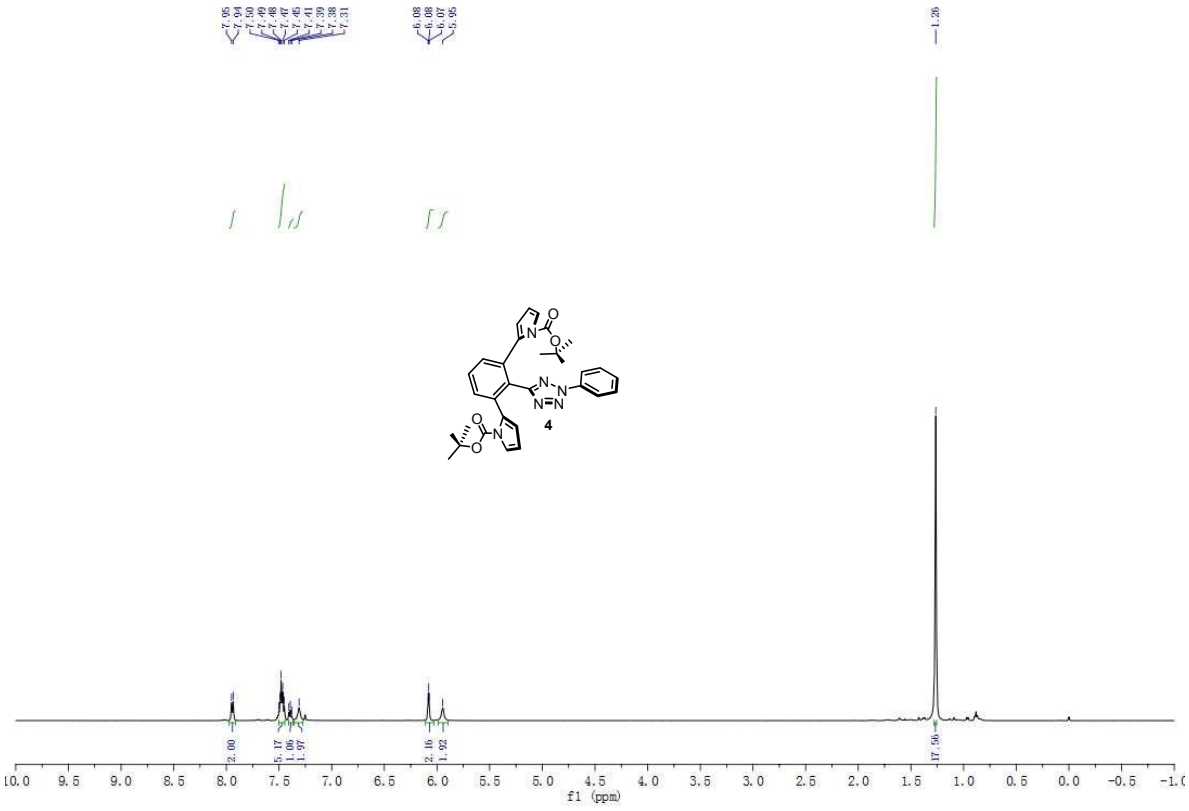
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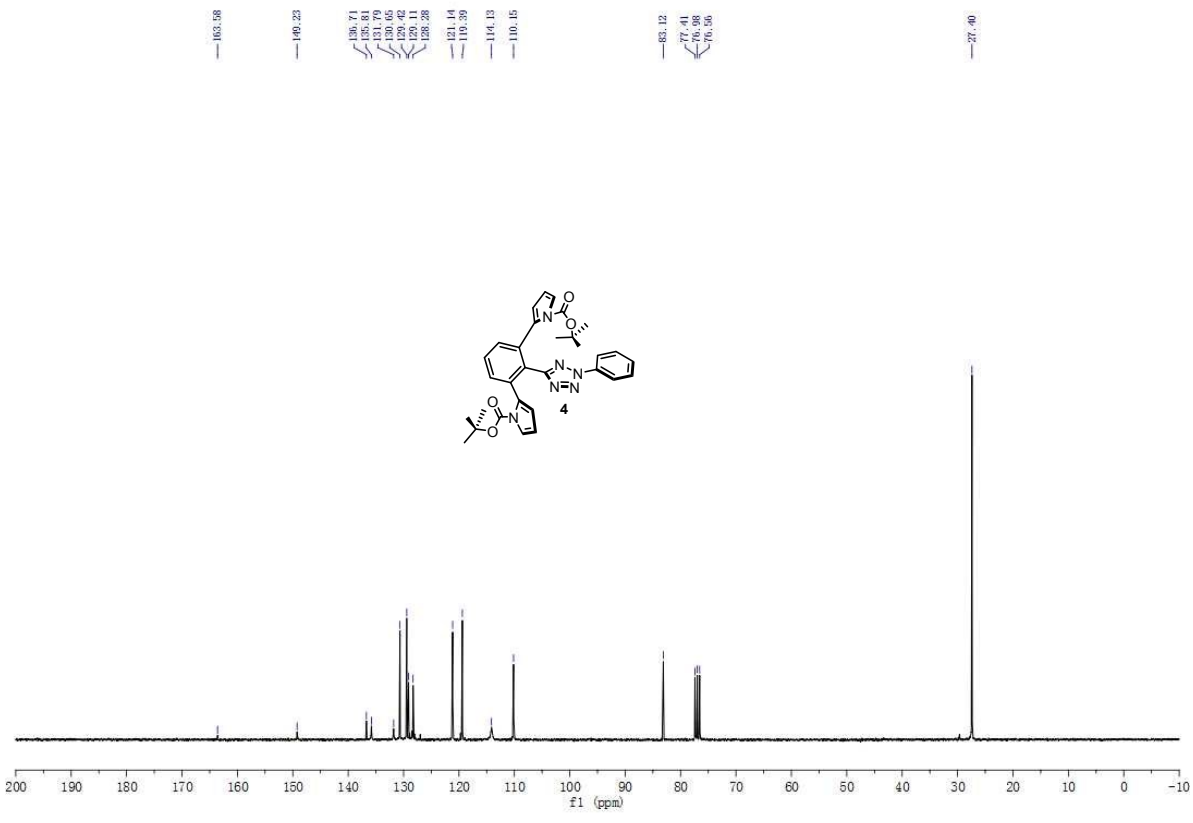
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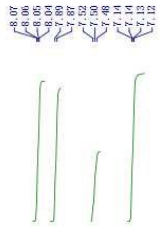
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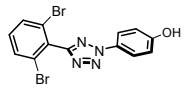
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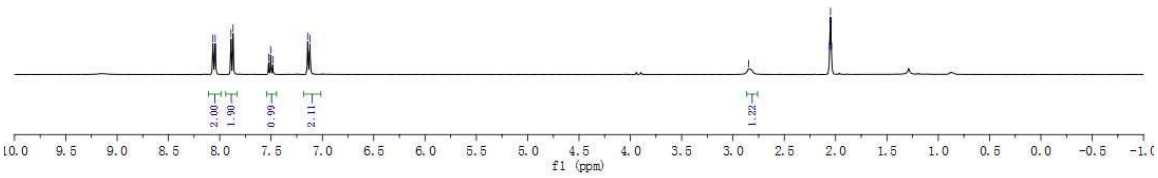
5b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f1 (ppm)


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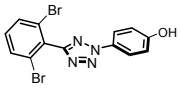
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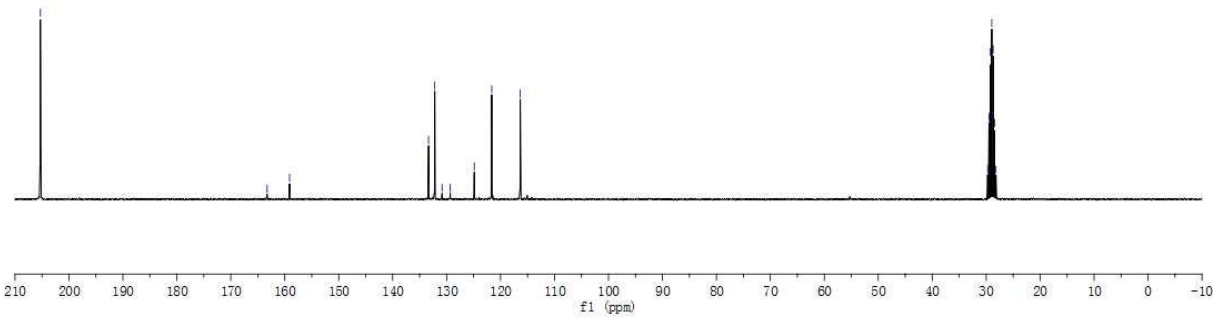
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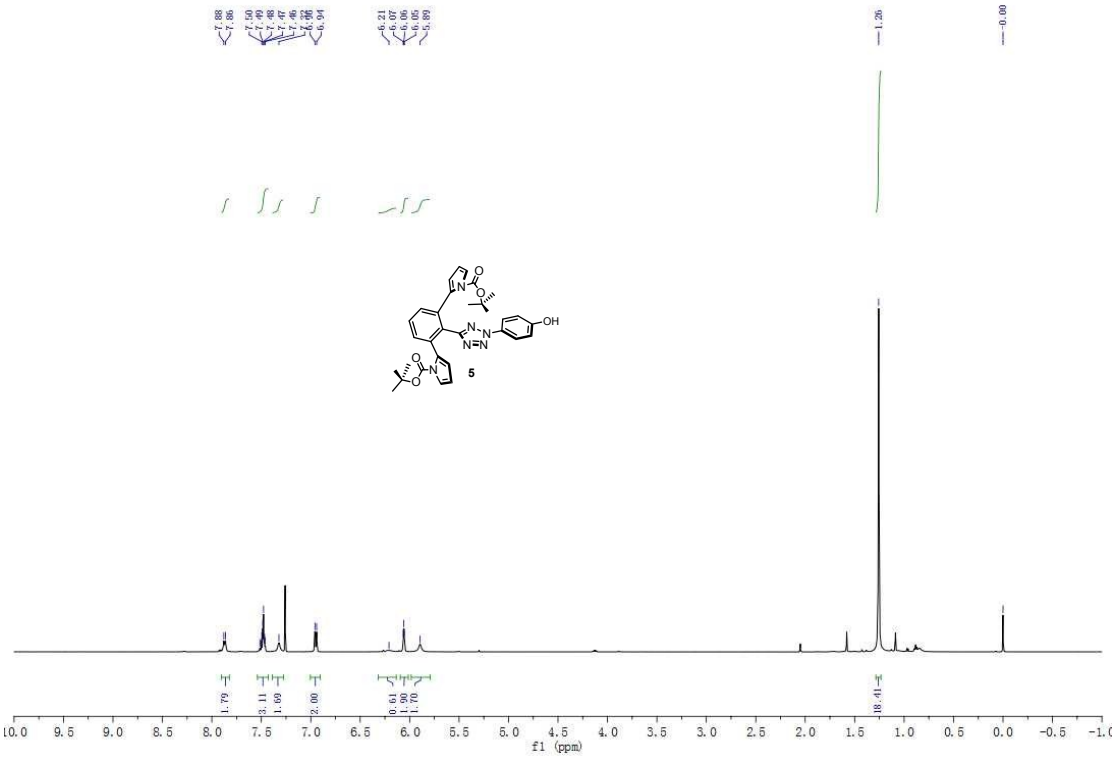
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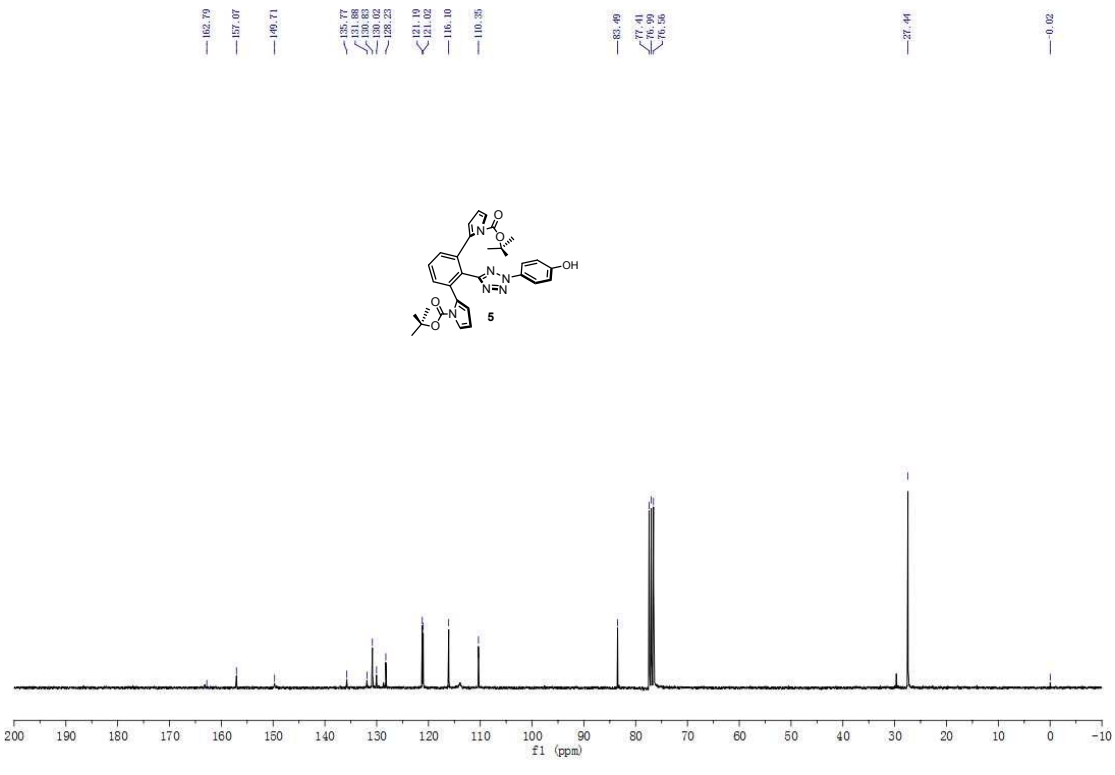
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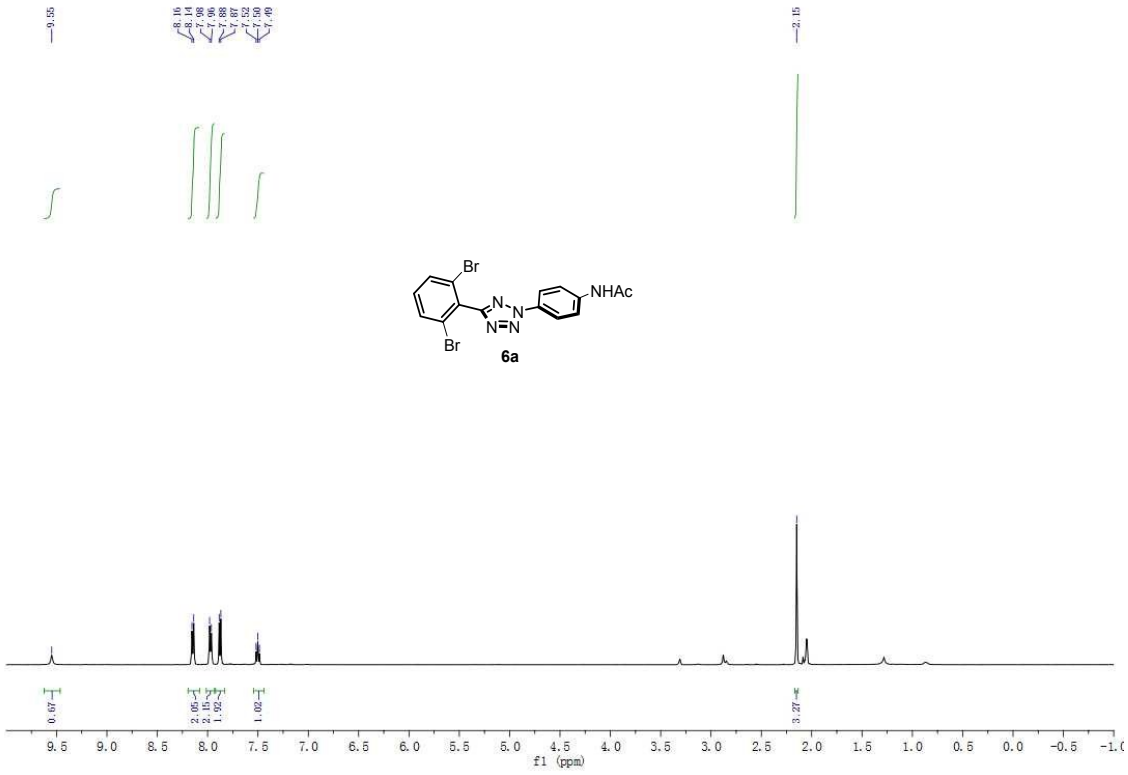
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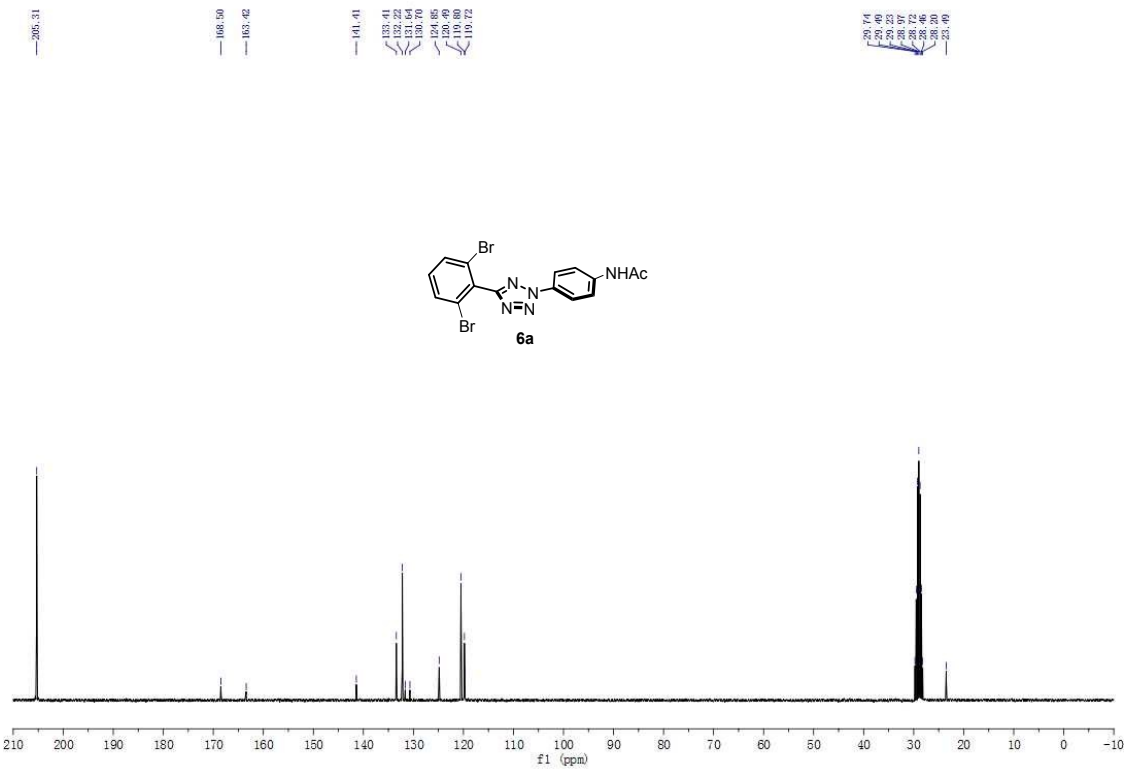
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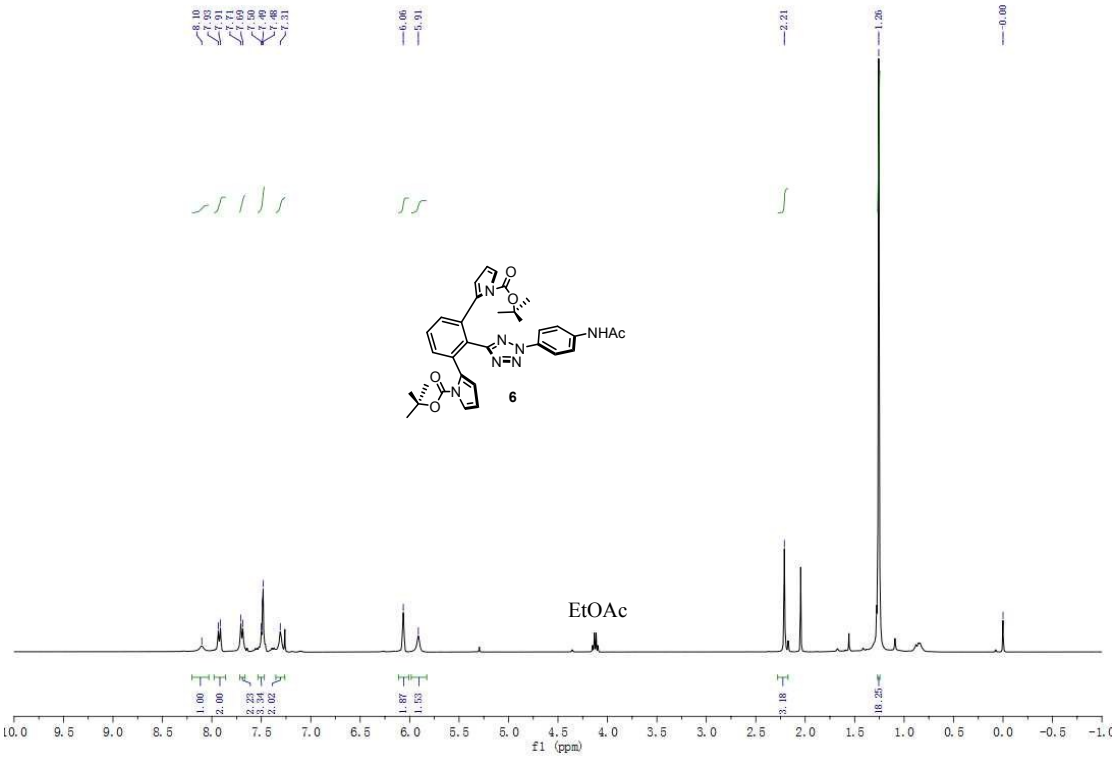
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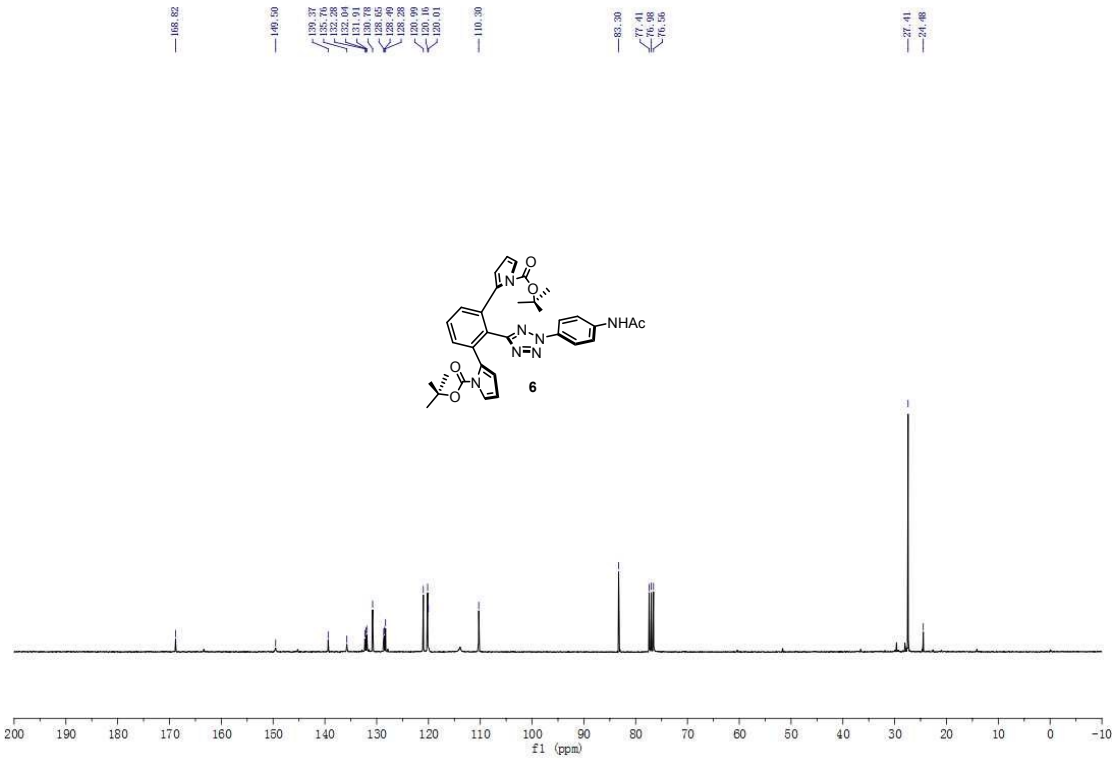
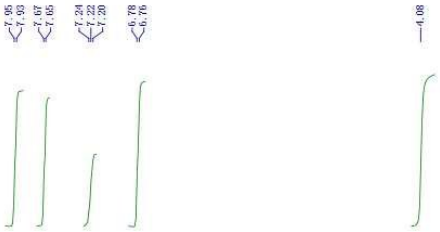
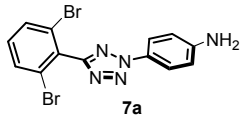
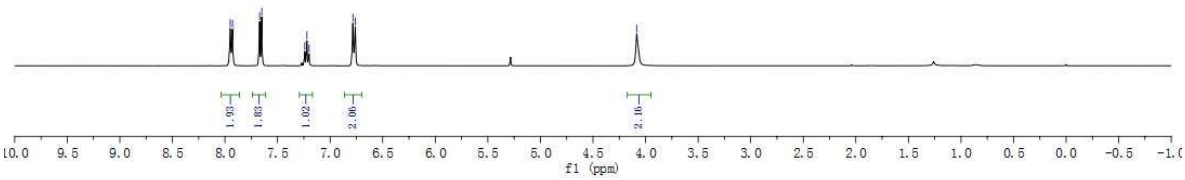
NHAc
6
EtOAc
1.00
2.00
2.23
3.34
2.02
1.87
1.53
3.18
18.25
f1 (ppm)


168.82
149.50
139.37
135.76
132.28
132.04
131.91
130.78
130.65
128.49
128.28
120.99
120.16
120.01
110.30
83.30
77.41
76.98
76.56
27.41
24.48
NHAc
6
f1 (ppm)

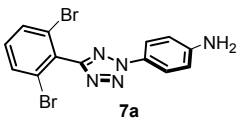

7.95
7.93
7.67
7.65
7.24
7.22
7.20
6.78
6.76
4.08


Br
NH2
Br

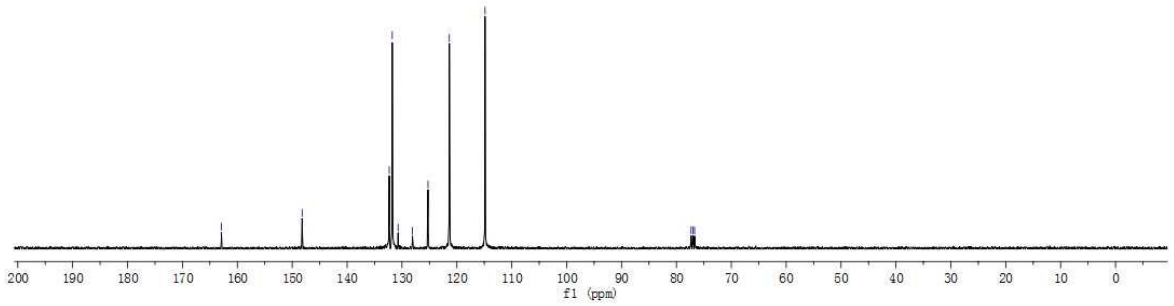
7a

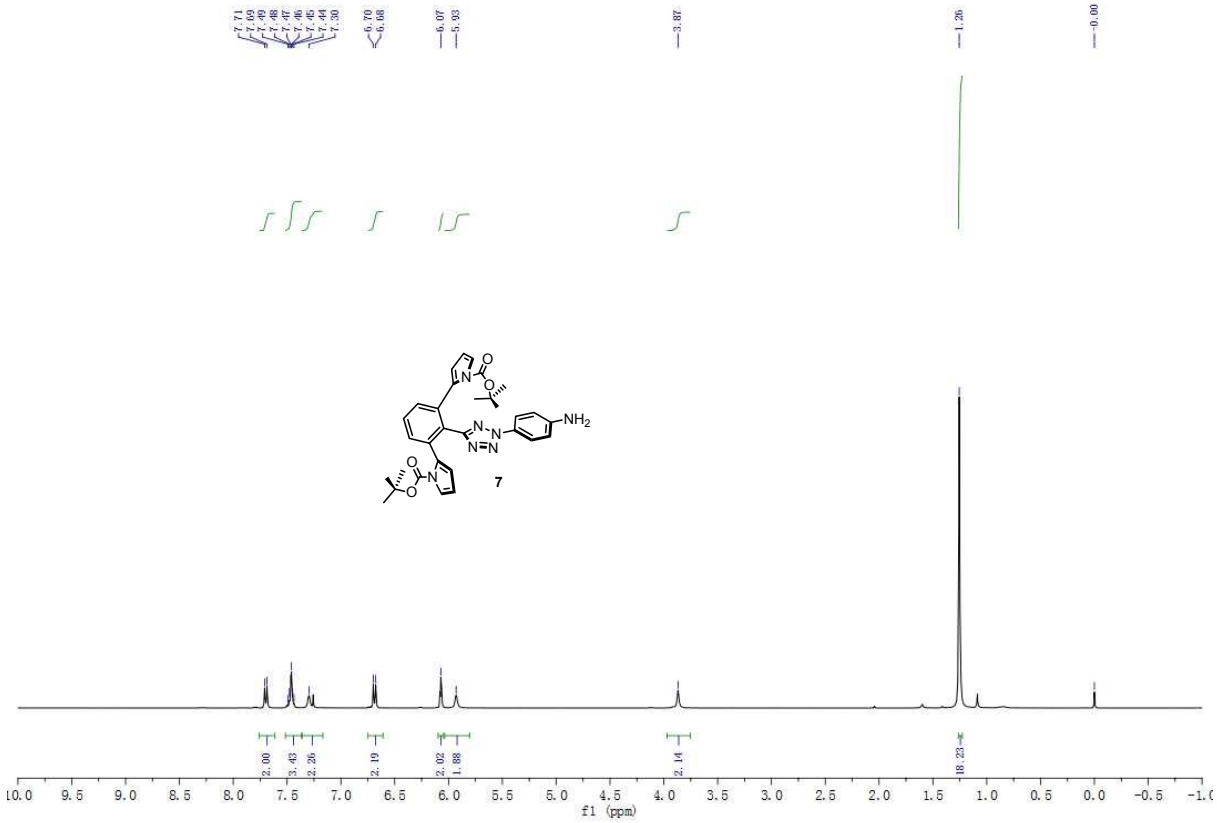

1.93
1.83
1.02
2.06
2.16
f1 (ppm)

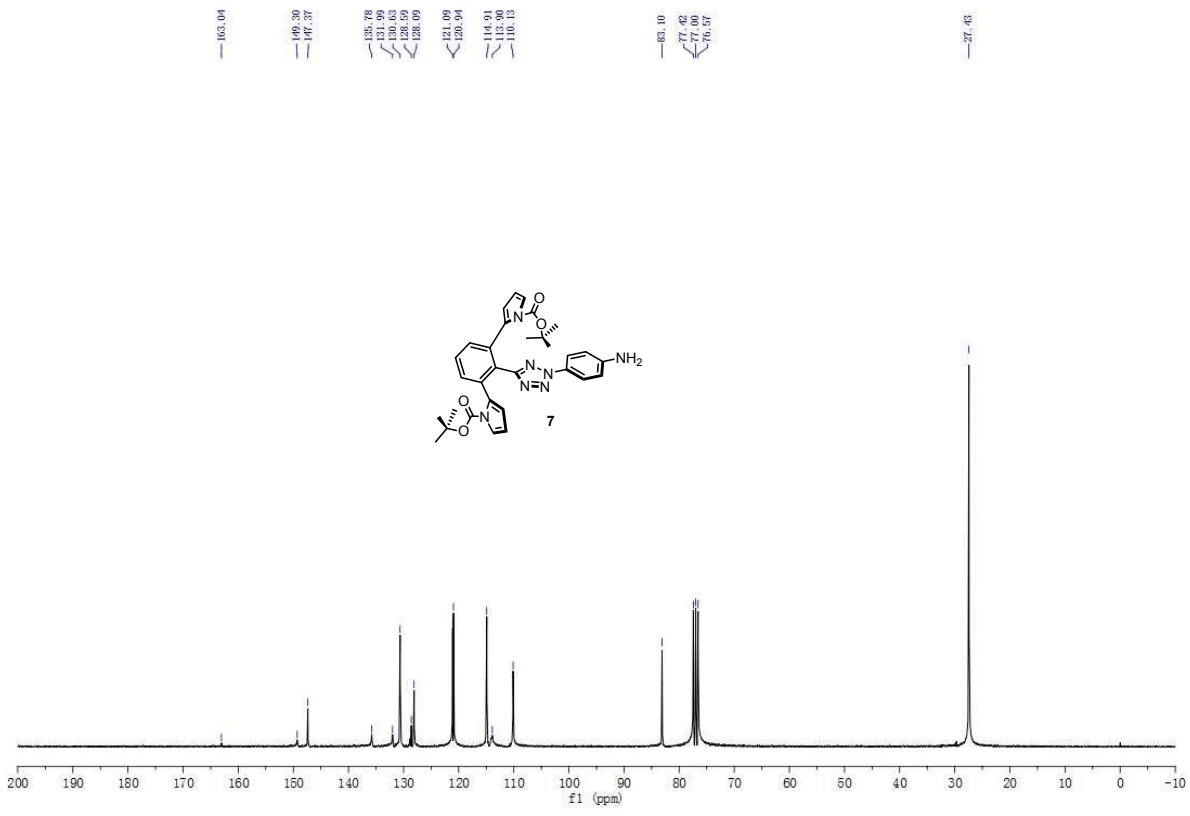

162.99
148.19
148.13
132.32
131.77
130.71
128.10
125.26
121.37
114.87
77.33
77.01
76.69

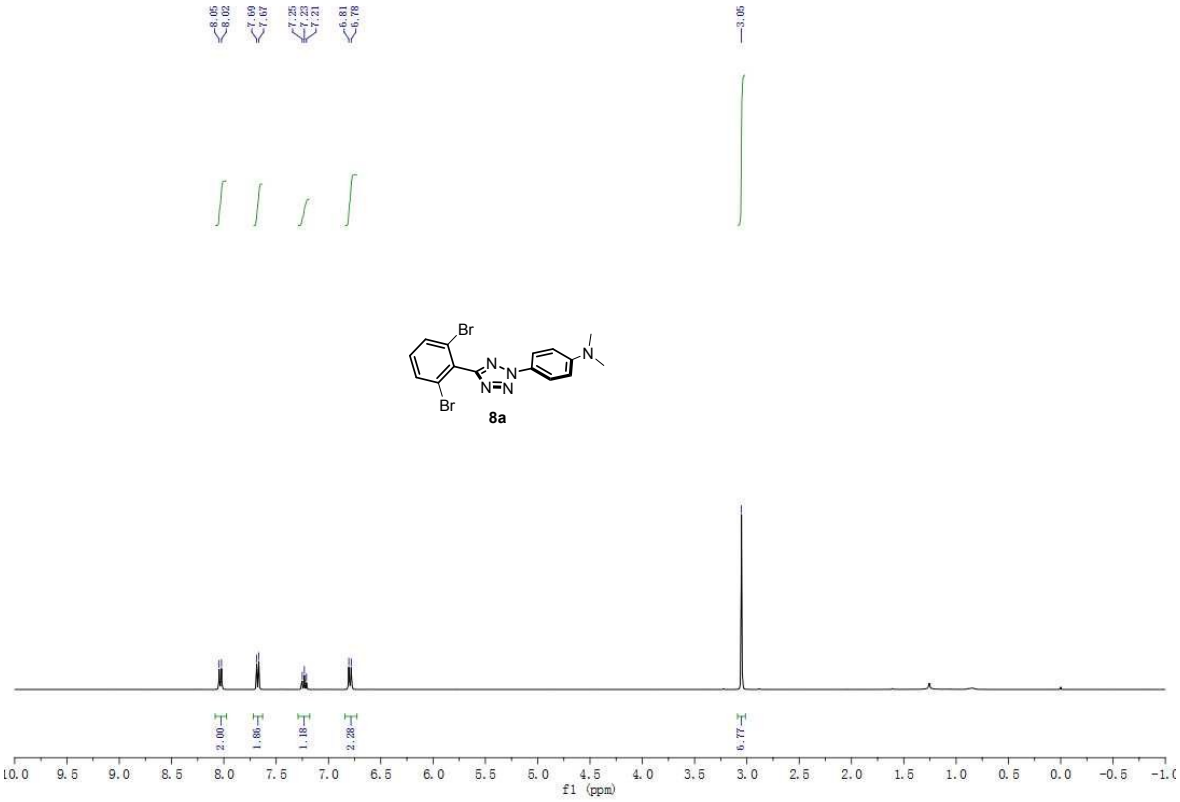

Br
NH2
Br

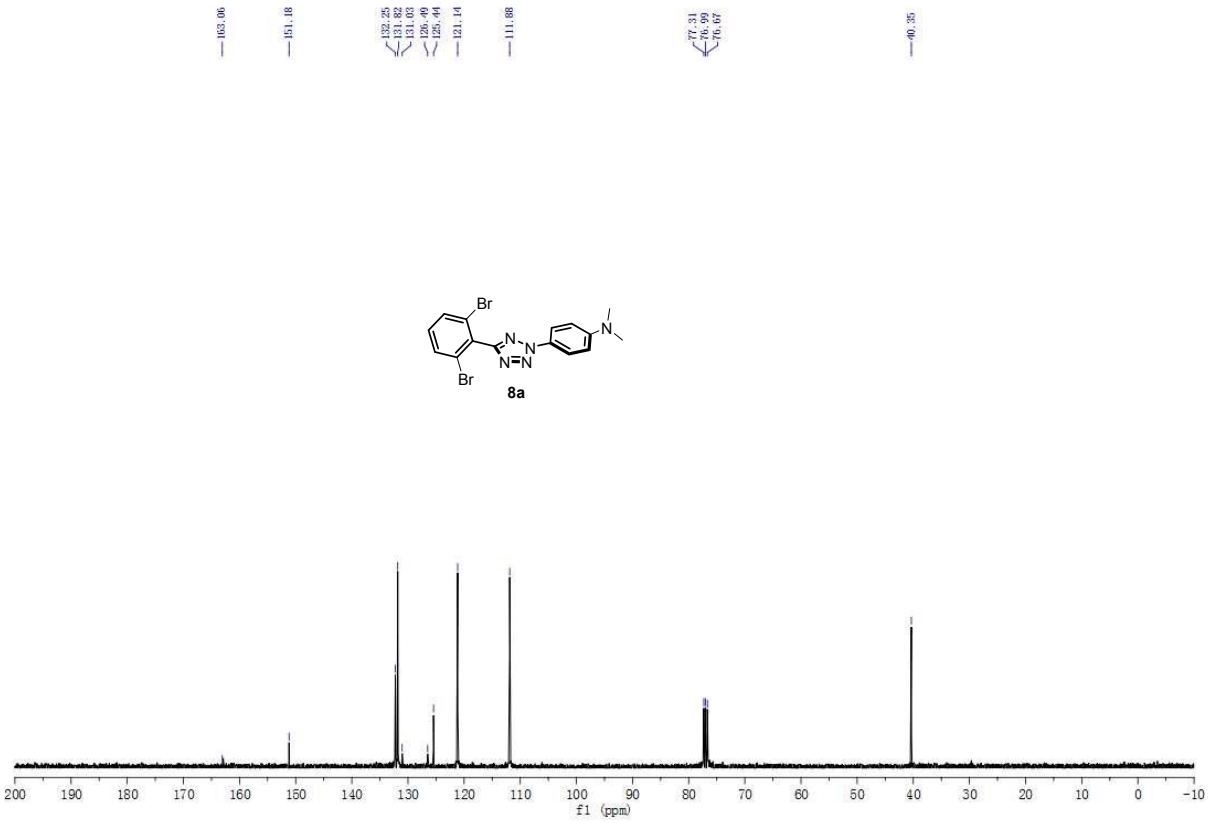
7a

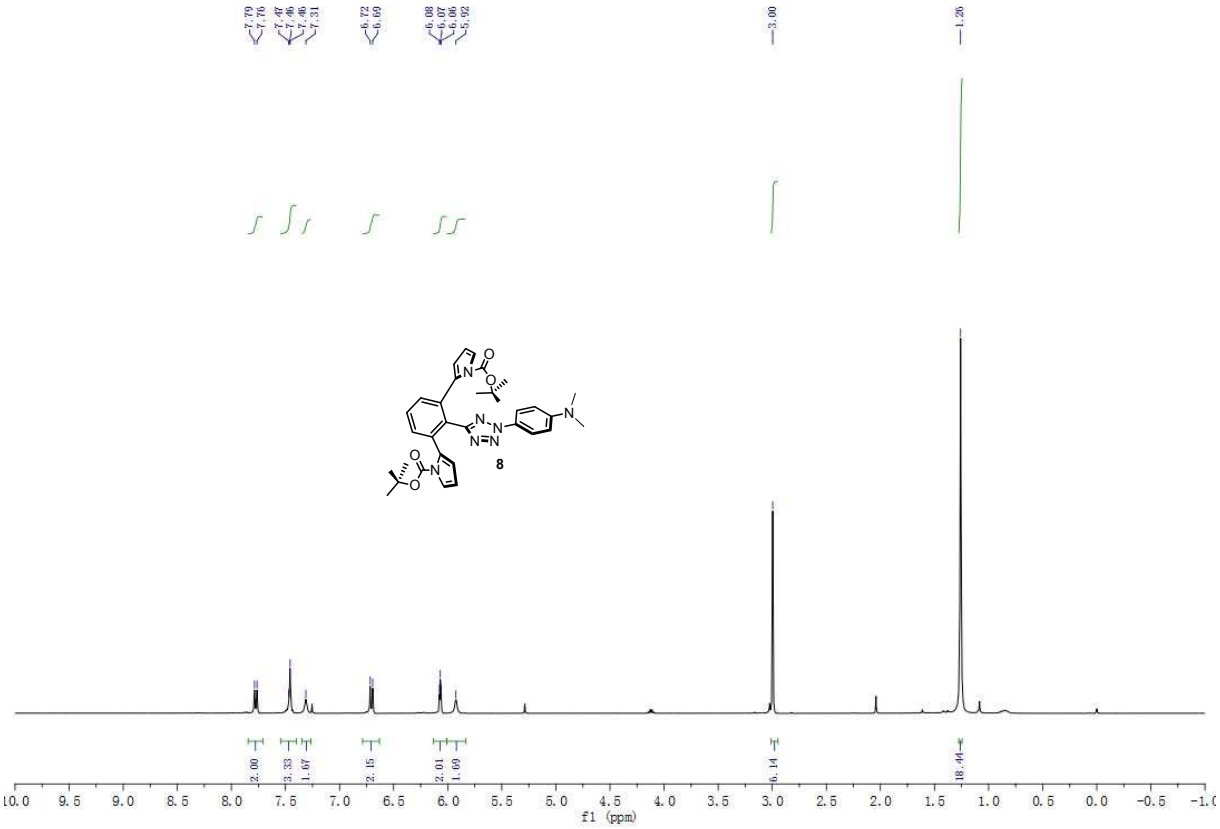

f1 (ppm)

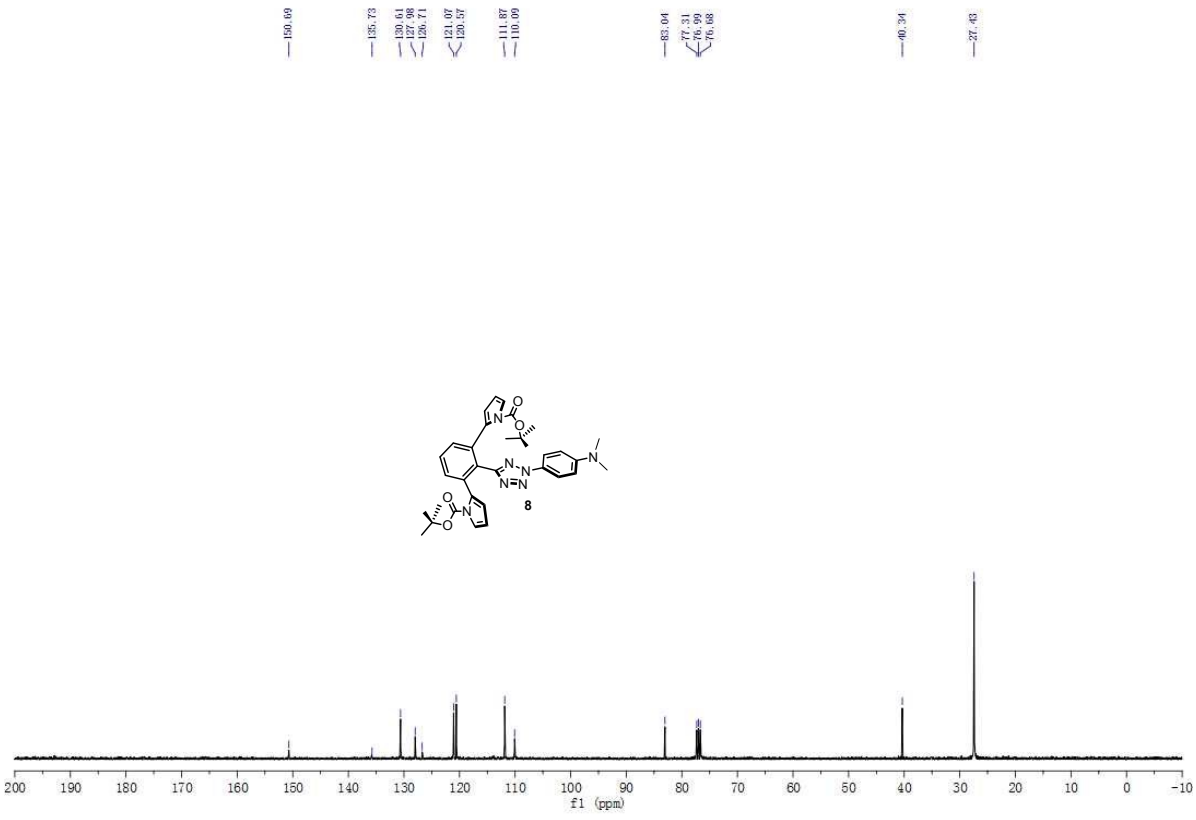

7.71
7.69
7.49
7.48
7.47
7.46
7.45
7.44
7.30
6.70
6.68
6.07
5.93
3.87
1.26
0.00
NH2
7
2.00
3.43
2.26
2.19
2.02
1.88
2.14
18.23
f1 (ppm)

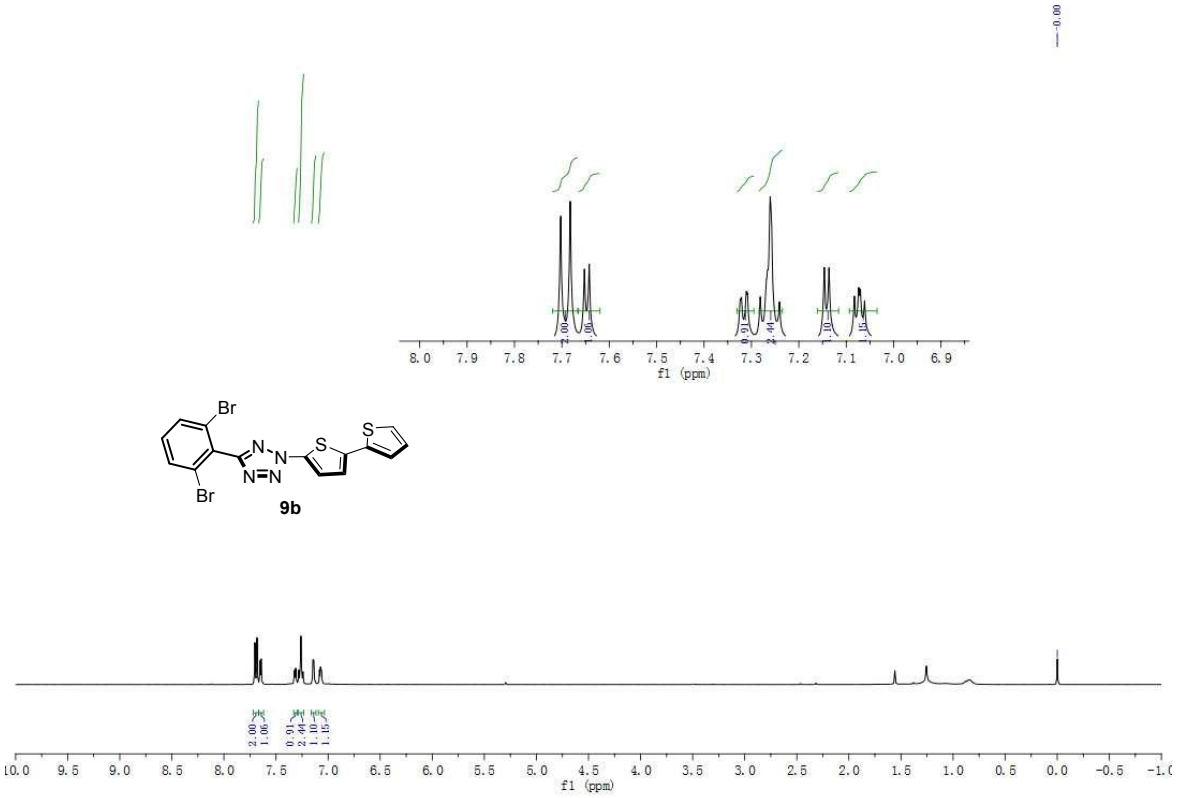

163.04
149.30
147.37
135.78
131.99
130.63
128.59
128.09
121.09
120.94
114.91
113.90
110.13
83.10
77.42
77.00
76.57
27.43
NH2
7
f1 (ppm)

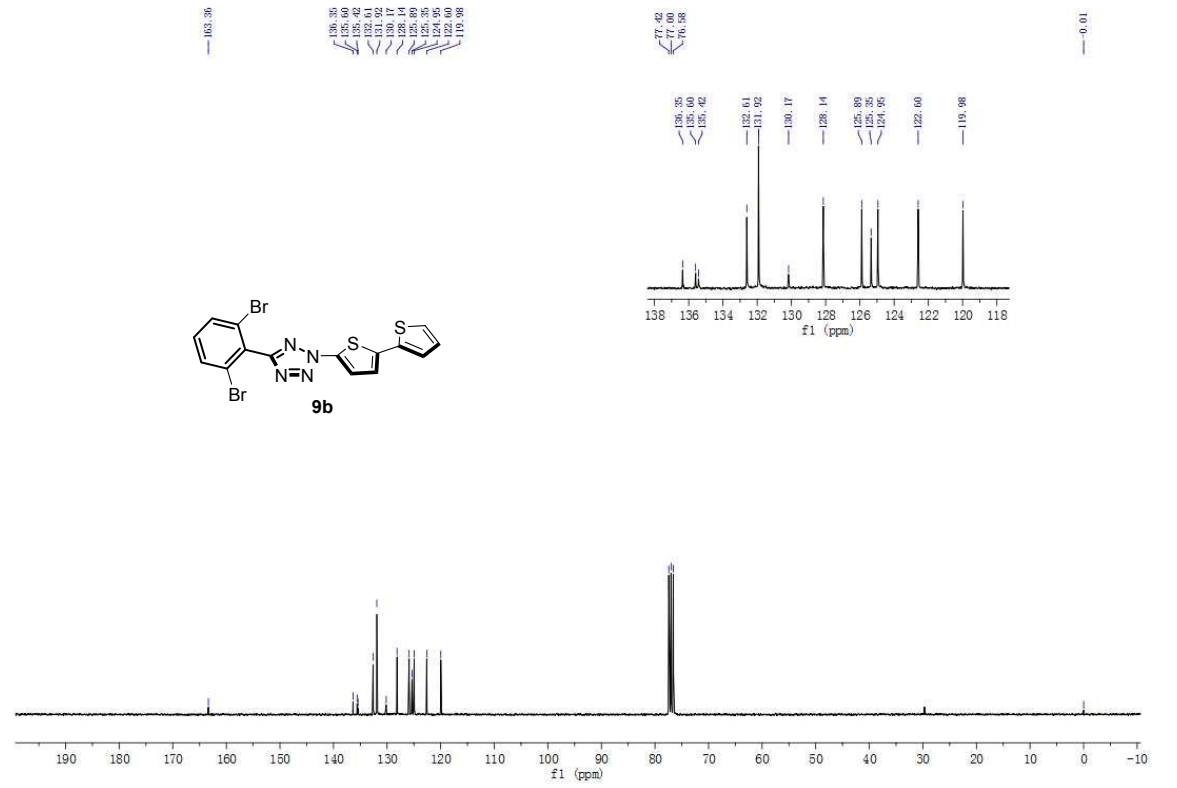

8.05
8.02
7.69
7.67
7.25
7.23
7.21
6.81
6.78
3.05
Br
Br
N
N
N
N
8a
2.00
1.86
1.18
2.28
6.77
f1 (ppm)

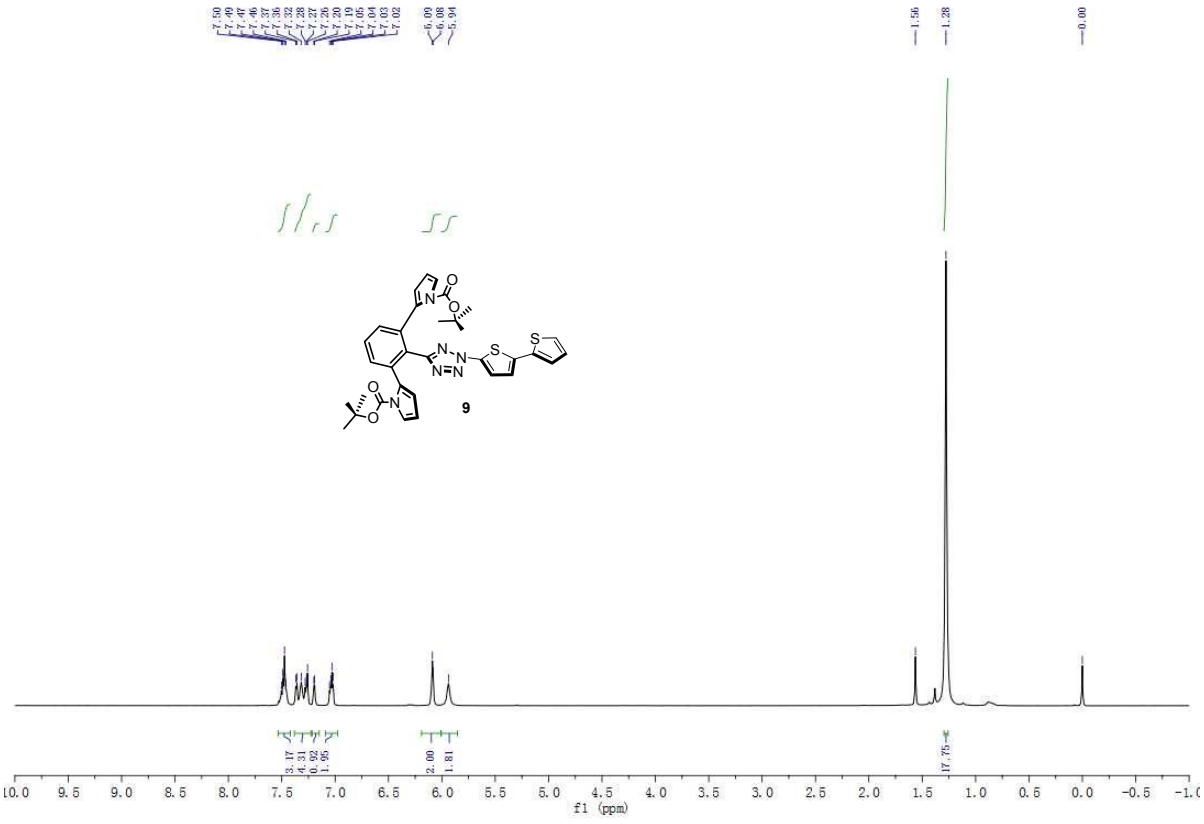

163.06
151.18
132.25
131.82
131.03
126.49
125.44
121.14
111.88
77.31
76.99
76.67
40.35
Br
Br
N
N
N
N
8a
f1 (ppm)

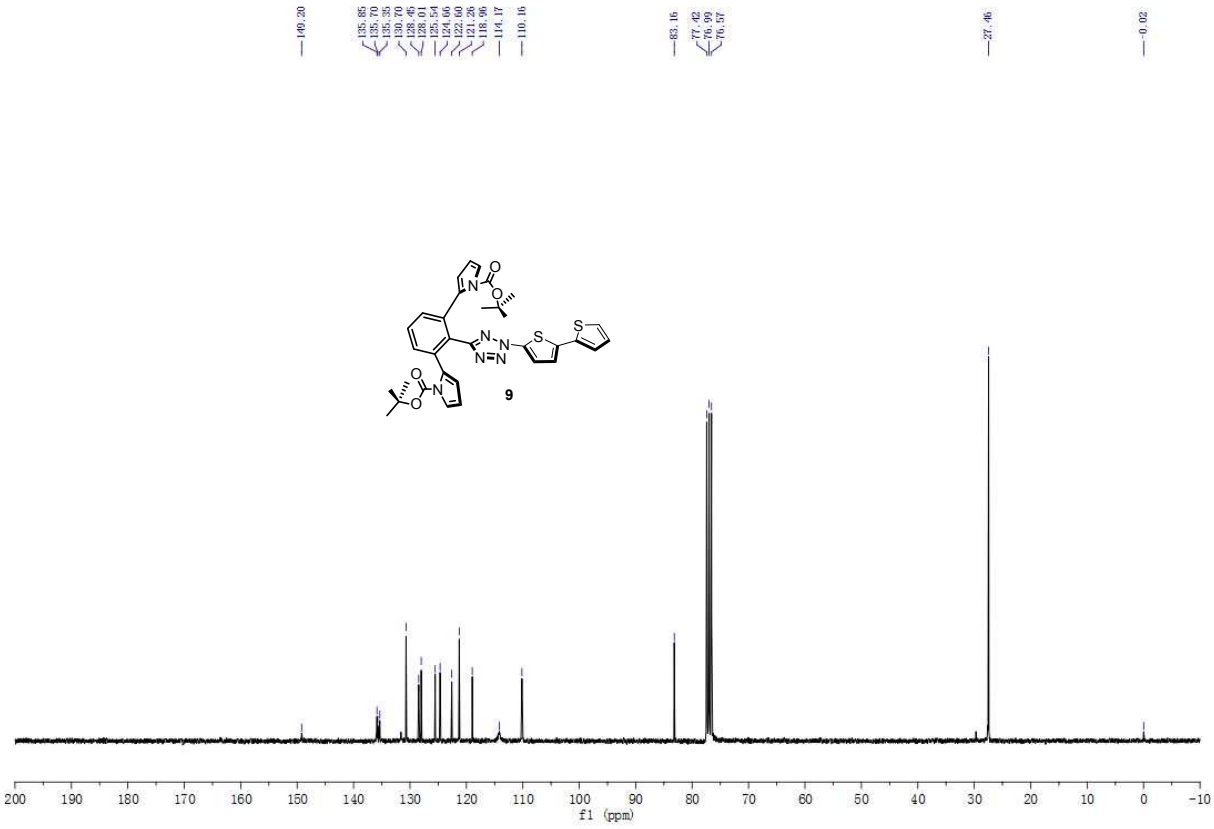

7.79
7.76
7.47
7.46
7.46
7.31
6.72
6.69
6.08
6.07
6.06
5.92
3.00
1.26
8
2.00
3.33
1.67
2.15
2.01
1.69
6.14
18.44
f1 (ppm)


150.69
135.73
130.61
127.98
126.71
121.07
120.57
111.87
110.09
83.04
77.31
76.99
76.68
40.34
27.43
8
f1 (ppm)


0.00
8.0 7.9 7.8 7.7 7.6 7.5 7.4 7.3 7.2 7.1 7.0 6.9
f1 (ppm)
Br
Br
N
N
N
S
S
9b
2.00
1.06
0.91
2.44
1.10
1.15
10.0 9.5 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 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)


163.36
136.35
135.60
135.42
132.61
131.92
130.17
128.14
125.89
125.35
124.95
122.60
119.98
77.42
77.00
76.58
0.01
138 136 134 132 130 128 126 124 122 120 118
f1 (ppm)
Br
Br
N
N
N
S
S
9b
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)


9
f1 (ppm)


9
f1 (ppm)