Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Hydrogen sulphide-triggered theranostic prodrugs based on dynamic chemistry of tetrazines

Marcelle D. Perretti, [a,b‡] Yaiza Pérez-Pérez, [a‡] Kevin Soler-Carracedo,[c] Endika Martín-Encinas,[d] Concepción Alonso,[*d] Jimena Scoccia[*,b] and Romen Carrillo[*a,b]

a.Instituto de Productos Naturales y Agrobiología (IPNA-CSIC), Avenida Astrofísico Francisco Sánchez 3, 38206, La Laguna, Spain.
b.Instituto Universitario de Bio-Orgánica "Antonio González", Universidad de La Laguna, Avenida Astrofísico Francisco Sánchez 2, P.O. Box 456, 38200, La Laguna, Spain.
c. Departamento de Física, Universidad de La Laguna, Apdo. 456, E-38200, San Cristóbal de La Laguna, Santa Cruz de Tenerife, Spain.
d.Department of Organic Chemistry I, Faculty of Pharmacy and Lascaray Research Center, University of Basque Country (UPV/EHU), 01006 Vitoria-Gasteiz, Spain.

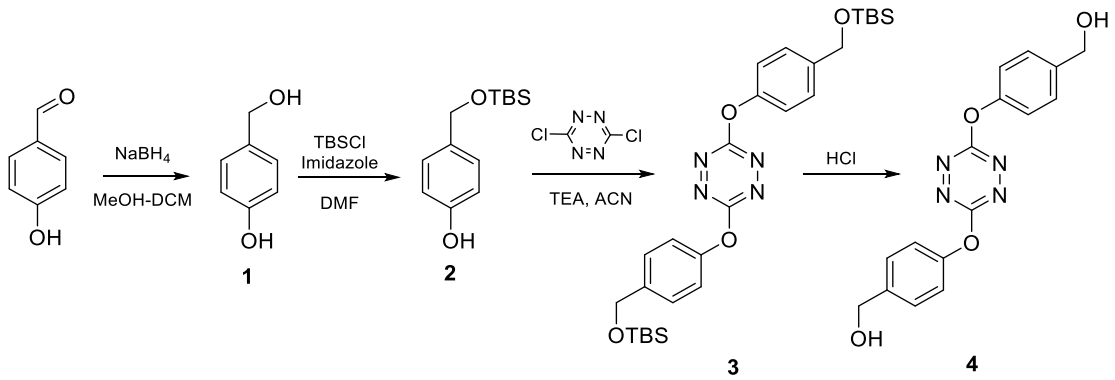
Table of Contents

1. Experimental Section

1.1 General information and materials

All reagents from commercial suppliers were used without further purification. All solvents were freshly distilled before use from appropriate drying agents. All other reagents were recrystallized or distilled when necessary. Reactions were performed under a dry nitrogen atmosphere. Analytical TLCs were performed with silica gel 60 F254 plates. Visualization was accomplished by UV light or vanillin with acetic and sulfuric acid in ethanol with heating. Column chromatography was carried out using silica gel 60 (230- 400 mesh ASTM). ^{1}H NMR spectra were recorded at 500 MHz and 400MHz, ^{13}C NMR spectra were recorded at 126 MHz and 100 MHz. Chemical shifts were reported in units (ppm) by assigning TMS resonance in the ^{1}H NMR spectrum as 0.00 ppm ($CDCl_3$, 7.26 ppm; DMSO-*d6* 2.50 ppm). Data were reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q=quartet, dd = double doublet, ddd = double double doublet, m =multiplet and br = broad), coupling constant (J values) in Hz and integration. Chemical shifts for ^{13}C NMR spectra were recorded in ppm from tetramethylsilane using the central peak of $CDCl_3$ (77.14 ppm) as the internal standard. High resolution mass spectra (HRMS) was measured by ESI method with an Agilent LC-Q-TOF-MS 6520 spectrometer. The emission spectra were recorded with an FSL 1000 fluorometer (Edinburgh Instruments) connected to a high-gain photomultiplier (PMT-900) detector. As an excitation source, a 450 W continuous Xenon lamp was used exciting at 370 nm. UV-Vis spectroscopy was performed on a Jasco-560 spectrophotometer.

1.2 Synthesis



Scheme S1 – Synthetic route to **4**.

Compound 1[1]**:**

To a solution of 4-hydroxybenzaldehyde (2 g, 16.4 mmol) in 100 mL of dry MeOH and 66 mL of dry CH_2Cl_2 at 0 °C was added $NaBH_4$ (37.8 mg, 1.0 mmol). The resulting mixture was then stirred at 0 °C for 15 min then 30 additional minutes at room temperature, under N_2. After completion of the reaction, the organic solvent was removed, water was added and the phases separated. The aqueous phase was extracted twice with ethyl acetate and the organic phases were combined, dried over Na_2SO_4 and evaporated to afford the 4-(hydroxymethyl)phenol **1** (1,78 g, 88%) as a white solid. The crude mixture was used for the subsequent step without any further purification.

Compound 2[2]**:**

4-(hydroxylmethyl)phenol **1** (1.47 g, 11.9 mmol) was dissolved in dry DMF (40 mL), then cooled in an ice-water bath. Imidazole (0.97 g, 14.2 mmol) and tertbutyldimethylsilyl chloride (2.14 g, 14.2 mmol) were added and the solution was left to stir overnight to room temperature. The reaction was diluted with ether (80 mL) and washed with saturated NH_4Cl (80 mL) and brine (40 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo to yield **2** as a yellow oil (1.82 g, 67%). The crude mixture was used for the subsequent step without any further purification.

Compound 3:

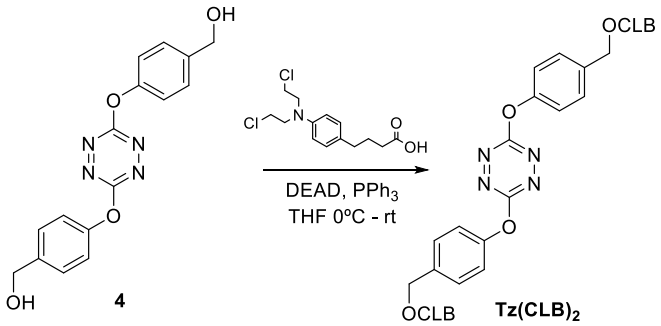
To a solution of 3,6-dichloro-1,2,4,5-tetrazine[3] (0.32 g, 2.1 mmol) in ACN (26 mL) was added the compound **2** (1.5 g, 6.3 mmol) and Et_3N (0.9 mL, 6.3 mmol) and the reaction mixture was stirred at room temperature for 1 h. The solvent was removed under vacuum and the afforded residue was purified by silica gel flash column chromatography using an elution of hexane/ethyl acetate (90:10), affording 0.75 g (64%) of a pink solid identified as **3**. ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 7.40 (d, *J* = 8.6 Hz, 4H), 7.22 (d, *J* = 8.6 Hz, 4H), 4.76 (s, 4H), 0.95 (s, 18H), 0.11 (s, 12H).^{13}C NMR (126 MHz, $CDCl_3$, δ ppm): 167.53, 151.39, 139.97, 127.67, 120.70, 64.43, 26.08, -5.12. HR-MS (ESI+,m/z): [M+Na]+ = $C_{28}H_{42}N_4O_4NaSi_2$, calcd.: 577.2642; found 577.2646.

Compound 4:

To a solution of **3** (0,73 g, 1.32 mmol) in ACN (15 mL) was added 2.8 mL of HCl (1M) at room temperature for 1 h. The organic solvent was removed under vacuum and the reaction mixture was dissolved in EtOAc. The organic layer was dried over Na_2SO_4 and

concentrated in vacuo to afford the compound **4** as a orange solid (0.40g, 93%). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): δ 7.43 (d, J = 8.3 Hz, 4H), 7.31 (d, J = 8.4 Hz, 4H), 5.26 (t, J = 5.7 Hz, 2H), 4.53 (d, J = 5.7 Hz, 4H).^{13}C NMR (126 MHz, DMSO-d_6, δ ppm): 166.87, 151.38, 140.44, 127.98, 120.22, 62.29. HR-MS (ESI+,m/z): [M+Na]+ = $C_{16}H_{14}N_4O_4Na$, calcd.: 349.0913; found 349.0917.

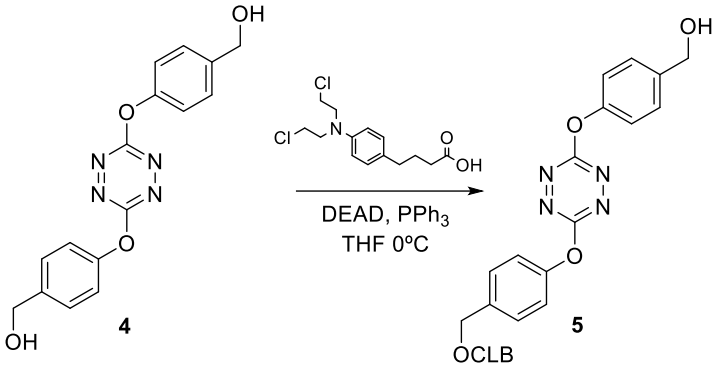
Synthesis of Tz(CLB)$_2$



Scheme S2 – Synthesis of **Tz(CLB)$_2$**.

To solution of **4** (0.05 g, 0.15 mmol), chlorambucil (0.103g, 0.34 mmol) and PPh_3 (0.104g, 0.4 mmol) in THF (5 mL) at 0ºC, DEAD (0.17 ml, 0.4 mmol) was added and the reaction mixture was stirred at room temperature for 30 minutes. The solvent was removed under vacuum and the afforded residue was purified by silica gel flash column chromatography using an elution of hexane/DCM (10:90), affording 0.085 g (65%) of a pink oil identified as **Tz(CLB)$_2$**. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.45 (d, J = 8.6 Hz, 4H), 7.26 (d, J = 8.5 Hz, 4H), 7.05 (d, J = 8.6 Hz, 4H), 6.62 (d, J = 8.7 Hz, 4H), 5.12 (s, 4H), 3.72 – 3.67 (m, 8H), 3.63 – 3.59 (m, 8H), 2.56 (t, J = 7.5 Hz, 4H), 2.37 (t, J = 7.5 Hz, 4H), 1.96 – 1.90 (m, 4H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ 173.42, 167.43, 152.31, 144.37, 134.67, 130.21, 129.86, 121.11, 112.47, 65.41, 53.82, 40.60, 34.09, 33.72, 26.82. HR-MS (ESI+,m/z): [M+Na]+ = $C_{44}H_{48}N_6O_6Na^{35}Cl_3{}^{37}Cl$, calcd.: 921.2258; found 921.2271. Anal. Calcd. For $C_{44}H_{48}N_6O_6NaCl_2$: C, 58.81; H, 5.38; N, 9.35; O, 10.68; Cl, 15.78 Found: C,58.73; H,5.388; N, 8.988.

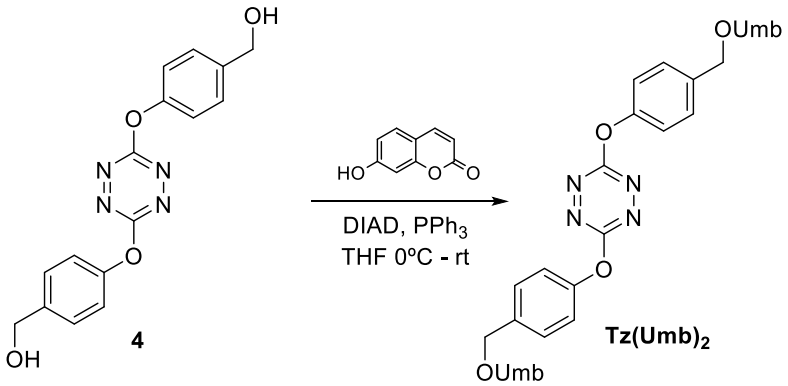
Synthesis of 5



Scheme S3 – Synthesis of **5**.

To solution of **4** (0.05 g, 0.15 mmol), chlorambucil (0.05 g, 0.17 mmol) and PPh_3 (0.051g, 0.2 mmol) in THF (5 mL) at 0ºC, DEAD (0.09 mL, 0.2 mmol) was added dropwise at 0°C and the reaction mixture was stirred for 1 h. The solvent was removed under vacuum and the afforded residue was purified by silica gel flash column chromatography using an elution of hexane/AcOEt (60:40), affording 0.05 g (28%) of a red solid identified as **5**. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.44 (t, *J* = 8.0 Hz, 4H), 7.25 (m, 4H), 7.03 (d, *J* = 8.6 Hz, 2H), 6.60 (d, *J* = 8.6 Hz, 2H), 5.11 (s, 2H), 4.72 (s, 2H), 3.72 – 3.64 (m, 4H), 3.63 – 3.56 (m, 4H), 2.54 (t, *J* = 7.5 Hz, 2H), 2.36 (t, *J* = 7.5 Hz, 2H), 1.95 – 1.88 (m, 2H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ 173.43, 167.51, 167.39, 152.32, 151.89, 144.46, 139.44, 134.62, 130.62, 130.21, 129.84, 128.74, 121.10, 121.05, 112.35, 65.41, 64.71, 53.74, 40.65, 34.06, 33.70, 26.81. HR-MS (ESI+,m/z): [M+Na]+ = $C_{30}H_{31}N_5O_5Na^{35}Cl_2$, calcd.: 634.1600; found 634.1591.

Syntheis of Tz(Umb)$_2$

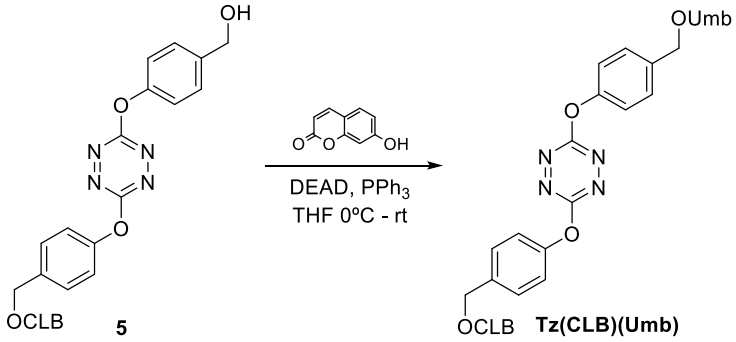


Scheme S4 – Synthesis of **Tz(Umb)$_2$**

To solution of **4** (0.05 g, 0.15 mmol), umbelliferone (Umb) (0.06 g, 0.34 mmol) and PPh_3 (0.1 g, 0.4 mmol) in THF (5 mL) at 0ºC, DIAD (0.08 mL, 0.4 mmol) was added and the reaction mixture was stirred at room temperature and inmediatly a orange precipitate started to appear. The reaction

mixture was filtered and washed with THF. The resulting pink solid (0.091 g, 48% yield) was was identified as **Tz(Umb)$_2$**. ^{1}H-NMR (400 MHz, DMSO-*d6*) δ 8.00 (d, *J* = 9.5 Hz, 2H), 7.65 (d, *J* = 8.6 Hz, 2H), 7.62 (d, *J* = 8.6 Hz, 4H), 7.42 (d, *J* = 8.6 Hz, 4H), 7.12 (d, *J* = 2.4 Hz, 2H), 7.05 (dd, *J* = 8.6, 2.4 Hz, 2H), 6.30 (d, *J* = 9.5 Hz, 2H), 5.26 (s, 4H). ^{13}C-NMR (126 MHz, DMSO-*d6*) δ 166.83, 161.36, 160.25, 155.33, 152.42, 144.29, 134.16, 129.83, 129.56, 120.73, 112.95, 112.66, 112.59, 101.65, 69.26. HR-MS (ESI+,m/z): [M+Na]+ = $C_{34}H_{22}N_4O_8Na$, calcd.: 637.1335; found 637.1345. Anal. Calcd. For $C_{34}H_{22}N_4O_8Na$: C, 66.45; H, 3.61; N, 9.12; O, 20.83 Found: C,66.52; H,3.620; N, 9.377.

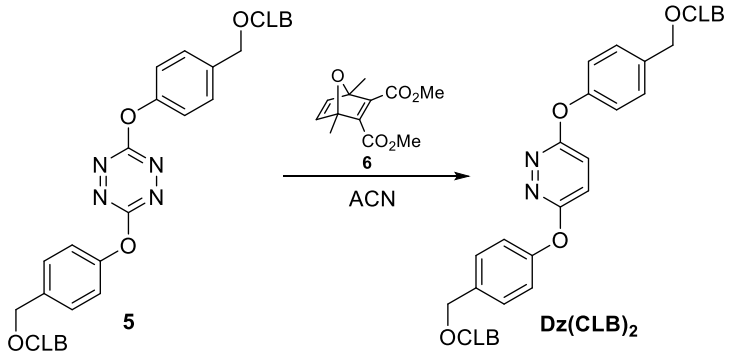
Synthesis of Tz(CLB)(Umb)



Scheme S5 – Synthesis of **Tz(CLB)(Umb)**

To solution of **5** (0.07 g, 0.11 mmol), umbelliferone (0.02 g, 0.13 mmol) and PPh_3 (0.038g, 0.15 mmol) in THF (5 mL) at 0ºC, DEAD (0.09 mL, 0.2 mmol) was added dropwise and the reaction mixture was stirred at room temperature for 1 h. The solvent was removed under vacuum and the afforded residue was purified by silica gel flash column chromatography using an elution of hexane/AcOEt (60:40), affording 0.04 g (50%) of a pink solid identified as **Tz(CLB)(Umb)**. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.64 (d, *J* = 9.5 Hz, 1H), 7.53 (d, *J* = 8.6 Hz, 2H), 7.45 (d, *J* = 8.6 Hz, 2H), 7.39 (d, *J* = 8.4 Hz, 1H), 7.31 (d, *J* = 8.6 Hz, 2H), 7.26 (d, *J* = 8.5 Hz, 2H), 7.05 (d, *J* = 8.6 Hz, 2H), 6.95 – 6.87 (m, 2H), 6.61 (d, *J* = 8.7 Hz, 2H), 6.27 (d, *J* = 9.5 Hz, 1H), 5.15 (s, 2H), 5.12 (s, 2H), 3.75 – 3.66 (m, 4H), 3.65 – 3.56 (m, 4H), 2.56 (t, *J* = 7.6 Hz, 2H), 2.37 (t, *J* = 7.5 Hz, 2H), 1.93 (m, 2H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ 173.41, 167.44, 167.41, 161.72, 161.21, 155.97, 152.40, 152.28, 144.45, 143.45, 134.67, 134.34, 130.64, 130.21, 129.84, 129.40, 129.04, 121.34, 121.10, 113.58, 113.28, 113.05, 112.36, 102.05, 69.81, 65.40, 53.75, 40.65, 34.07, 33.70, 26.81. (ESI+,m/z): [M+Na]+ = $C_{39}H_{35}N_5O_7Na^{35}Cl_2$, calcd.: 778.1811; found 778.1825. Anal. Calcd. For $C_{39}H_{35}N_5O_7Na^{35}Cl_2$: C, 61.91; H, 4.66; N, 9.26; O, 14.80; Cl, 9.37 Found: C,61.89; H,4.885; N, 9.553.

Synthesis of Dz(CLB)$_2$



Scheme S6 – Synthesis of **Dz(CLB)$_2$**

To a solution of **Tz(CLB)$_2$** (0.04 g, 0.045 mmol) in ACN (3 mL), dimethyl-1,4-dimethyl-7-oxabicyclo[2.2.1]hepta-2,5-diene2,3-dicarboxylate **6** (0.032 g, 0.13 mmol) was added and the reaction mixture was stirred at 80 ºC for 5 days. The reaction mixture solvent was removed under vacuum and the afforded residue was purified by silica gel flash column chromatography using an elution of hexane/AcOEt (70:30), affording 0.031 g (78%) of a oil identified as **Dz(CLB)$_2$** ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.37 (d, *J* = 8.5 Hz, 4H), 7.27 (d, 2H), 7.19 (d, *J* = 8.5 Hz, 4H), 7.06 (d, *J* = 8.6 Hz, 4H), 6.62 (d, *J* = 8.6 Hz, 4H), 5.09 (s, 4H), 3.70 (m, 8H), 3.62 (m, 8H), 2.56 (t, *J* = 7.5 Hz, 4H), 2.36 (t, *J* = 7.5 Hz, 4H), 1.93 (m, 4H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ 173.48, 163.15, 153.58, 144.46, 133.11, 130.65, 129.97, 129.84, 122.09, 121.37, 112.31, 65.68, 53.74, 40.67, 34.08, 33.75, 29.83, 26.83. (ESI+,m/z): [M+Na]+ = $C_{46}H_{50}N_4O_6Na^{35}Cl_3{}^{37}Cl$, calcd.: 919.2353; found 919.2377. Anal. Calcd. For $C_{46}H_{50}N_4O_6Cl_3Cl$: C, 61.61; H, 5.62; N, 6.25; O, 10.70; Cl, 15.81 Found: C,61.39; H, 5.978; N, 6.021.

2. NMR spectra

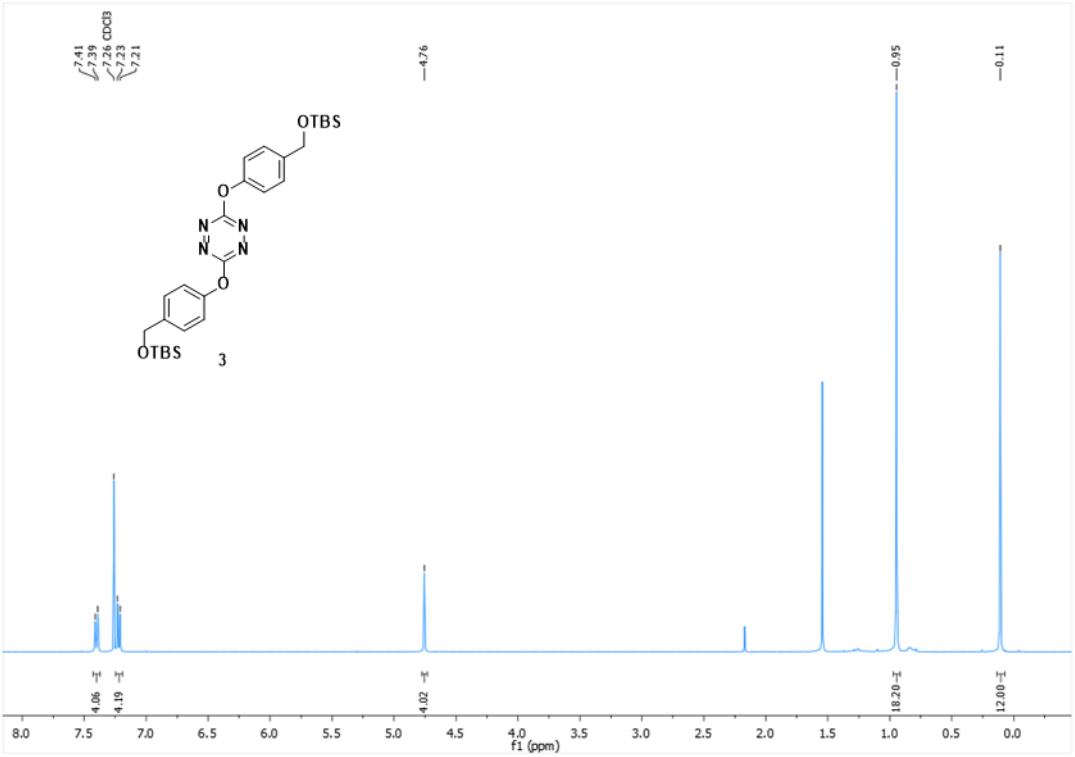


Fig. S1. ^{1}H-NMR spectrum (400 MHz, $CDCl_3$, 298 K) of 3.

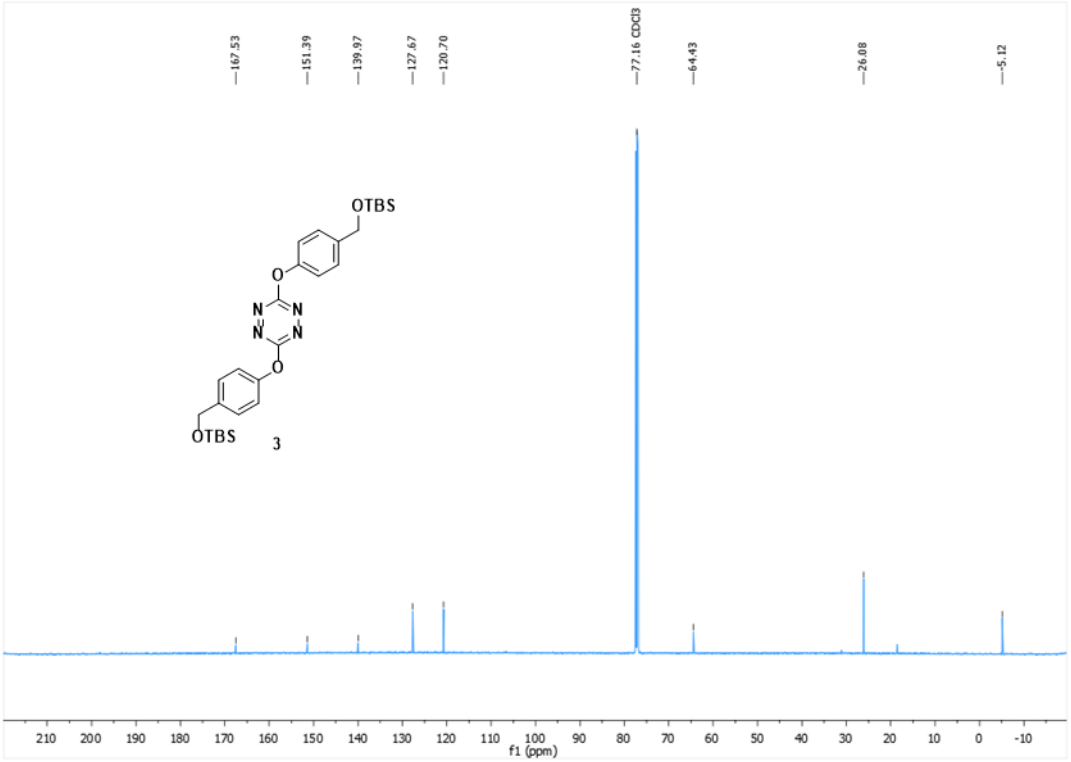


Fig. S2. ^{13}C-NMR spectrum (126 MHz, $CDCl_3$, 298 K) of 3.

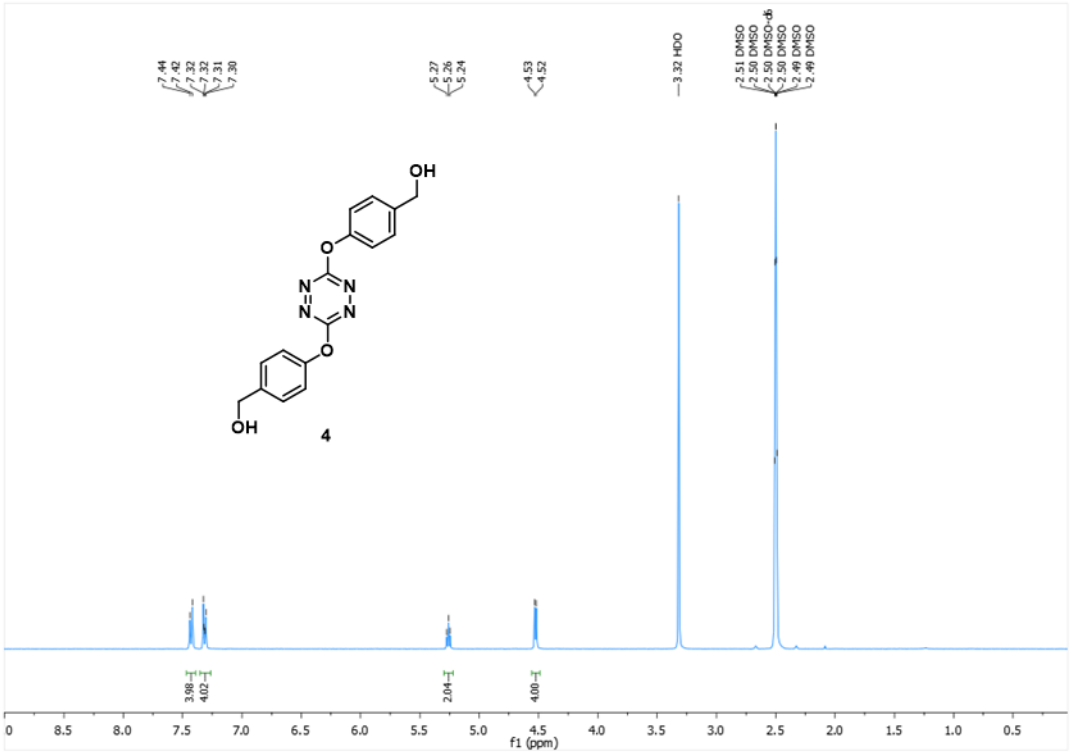


Fig. S3. ^{1}H-NMR spectrum (400 MHz, DMSO-*d*$_6$, 298 K) of 4.

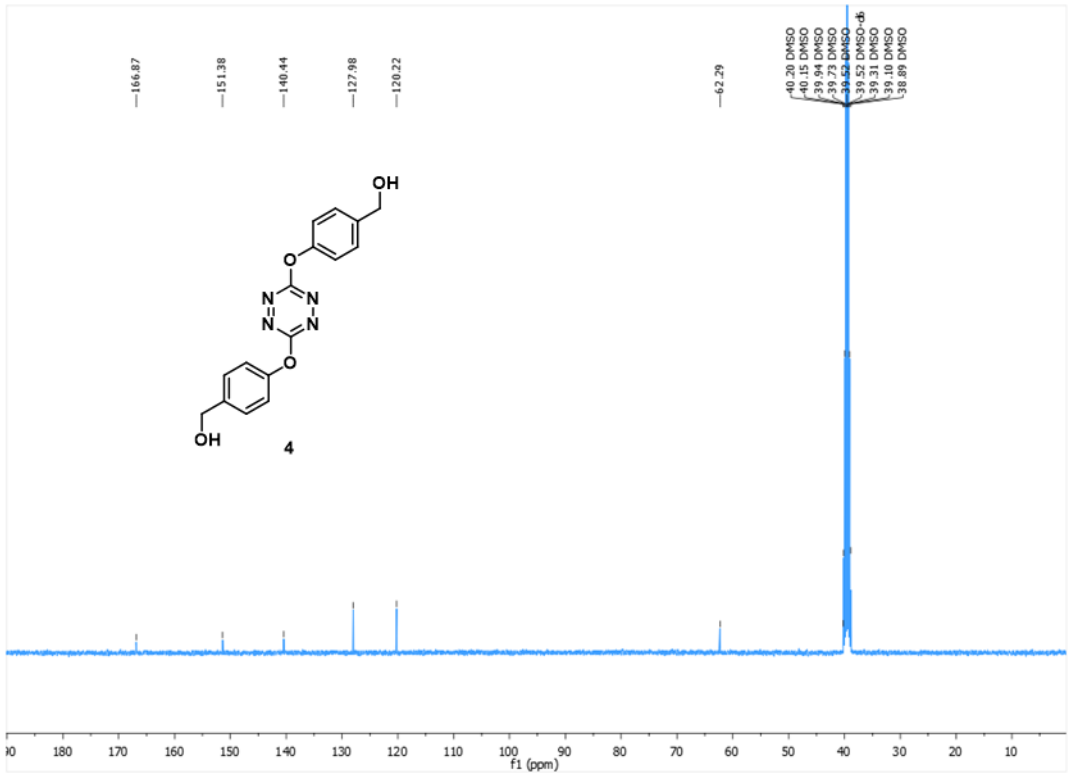


Fig. S4. ^{13}C-NMR spectrum (126 MHz, DMSO-*d*$_6$, 298 K) of 4.

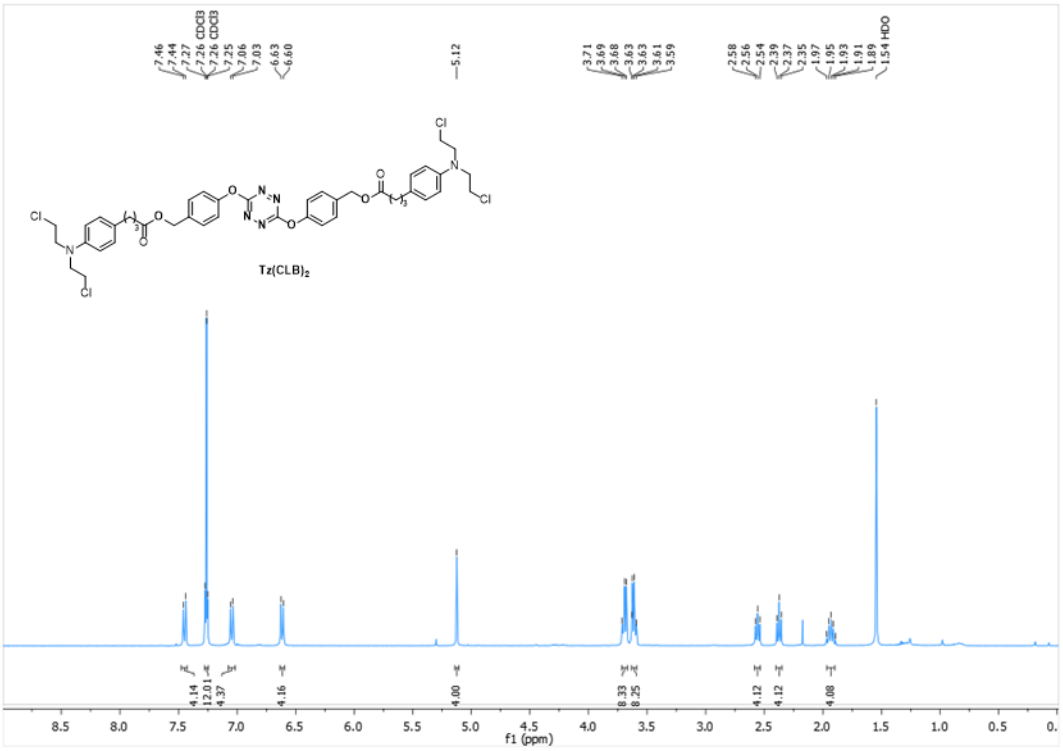


Fig. S5. ^{1}H-NMR spectrum (400 MHz, $CDCl_3$, 298 K) of $Tz(CLB)_2$.

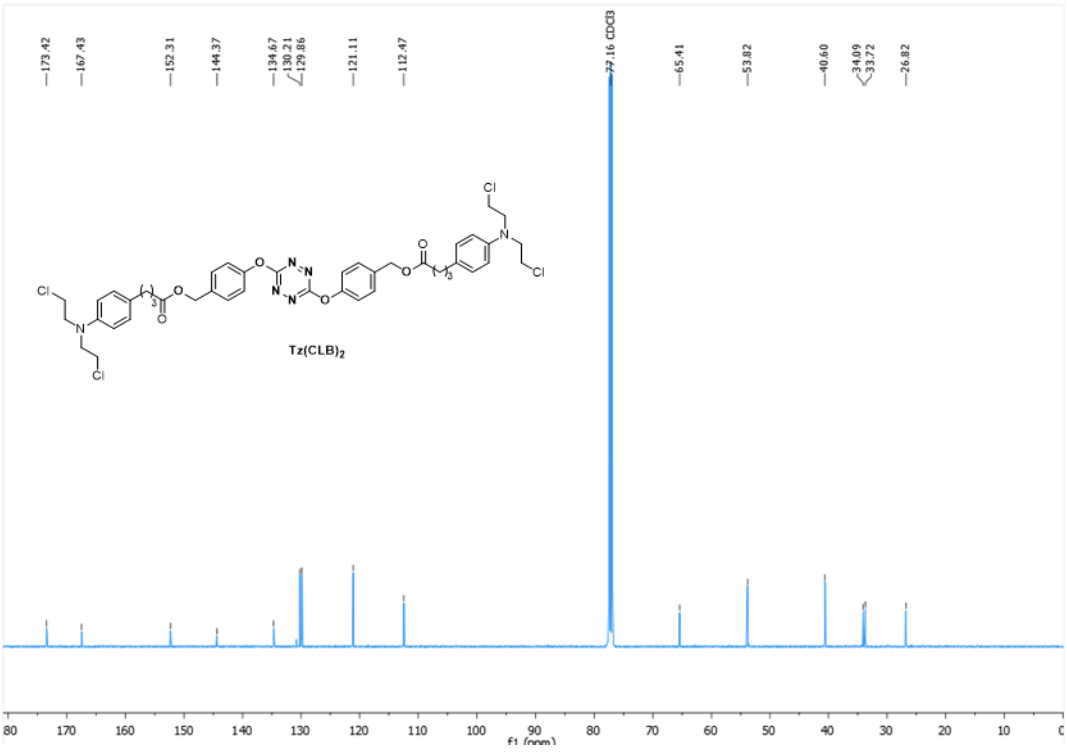


Fig. S6. ^{13}C-NMR spectrum (126 MHz, $CDCl_3$, 298 K) of $Tz(CLB)_2$.

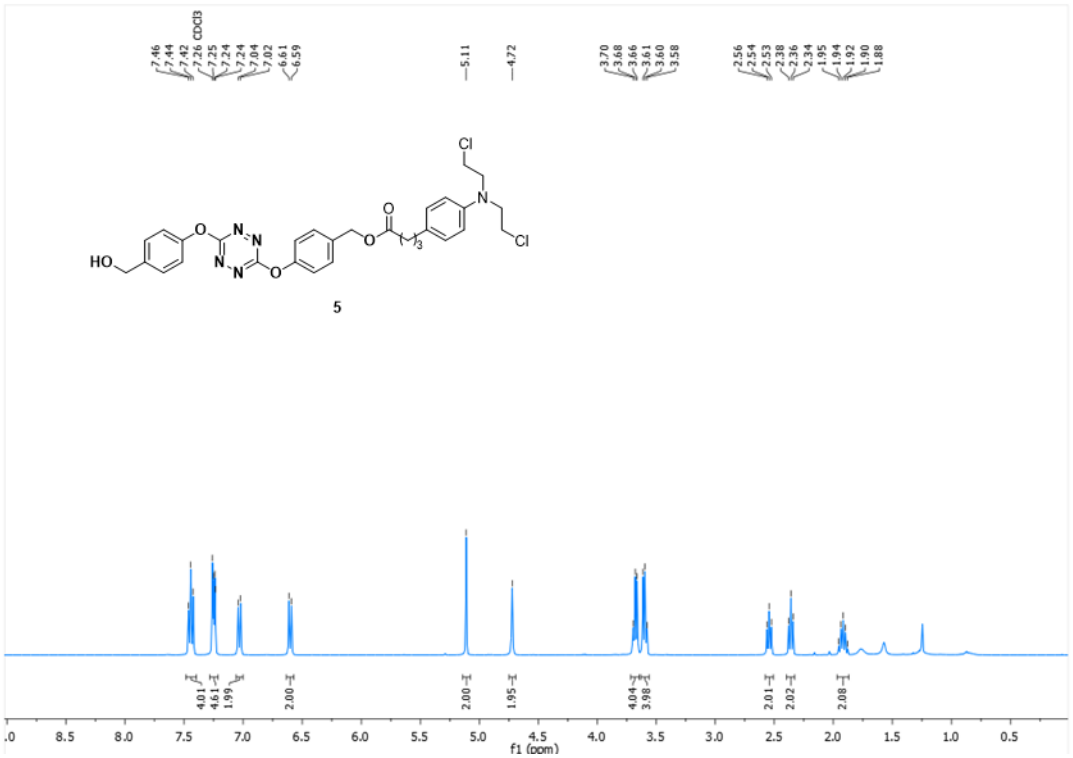


Fig. S7. ^{1}H-NMR spectrum (400 MHz, $CDCl_3$, 298 K) of 5.

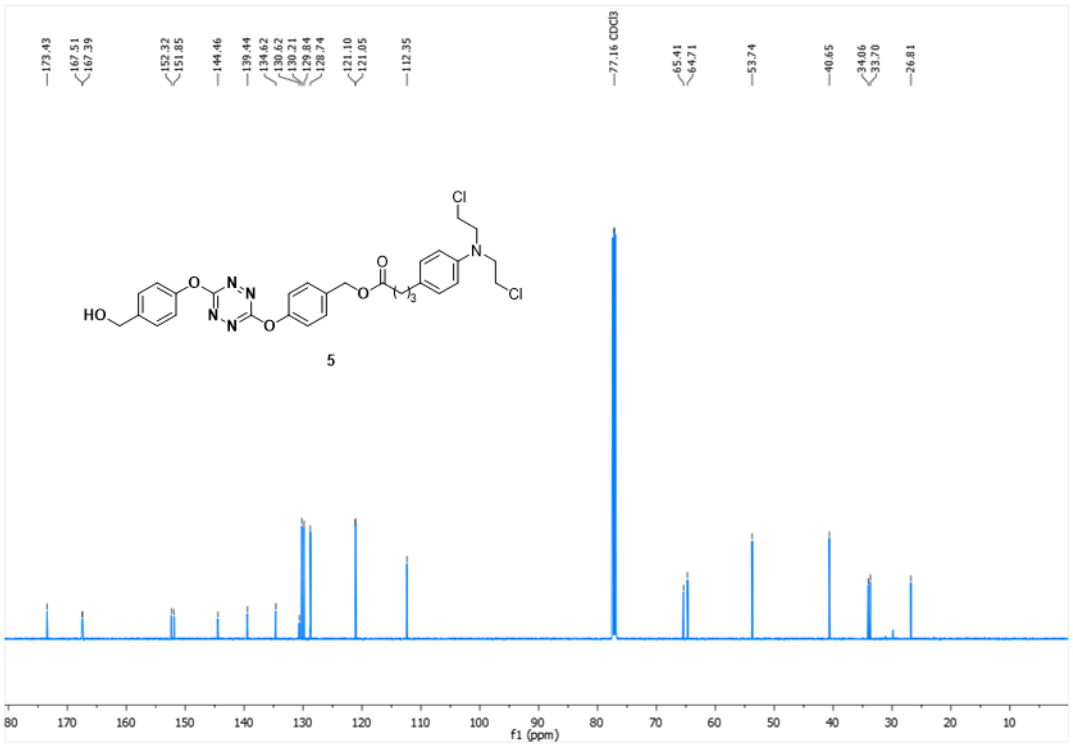


Fig. S8. ^{13}C-NMR spectrum (126 MHz, $CDCl_3$, 298 K) of 5.

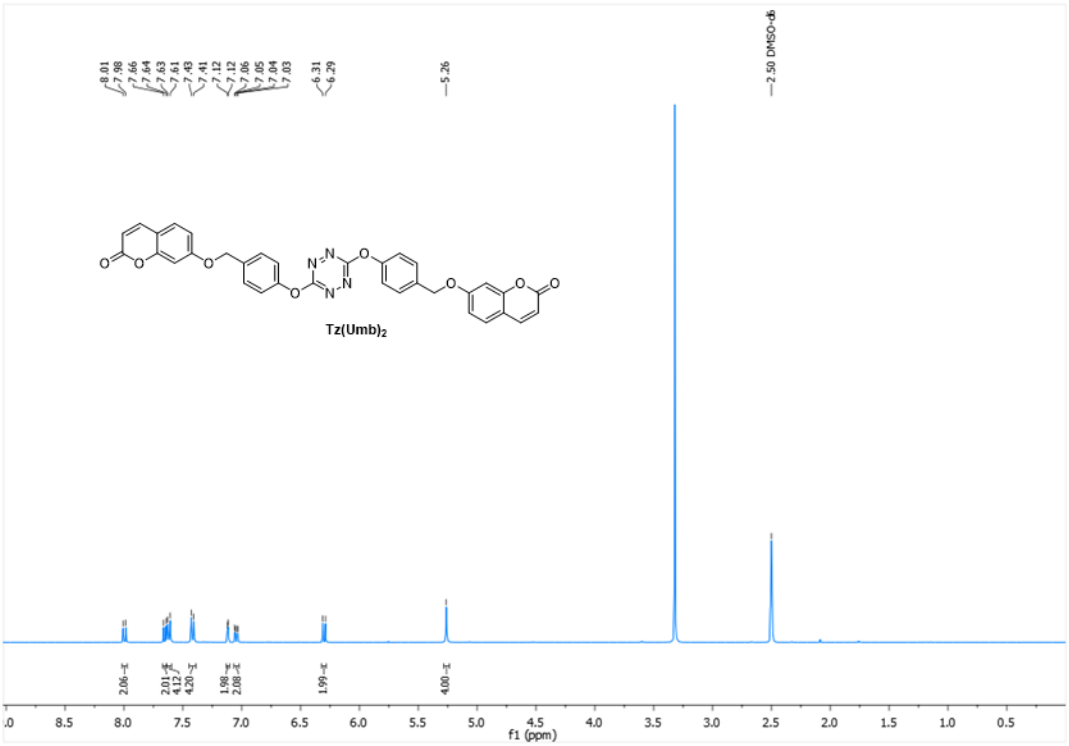


Fig. S9. ^{1}H-NMR spectrum (400 MHz, $CDCl_3$, 298 K) of $Tz(Umb)_2$.

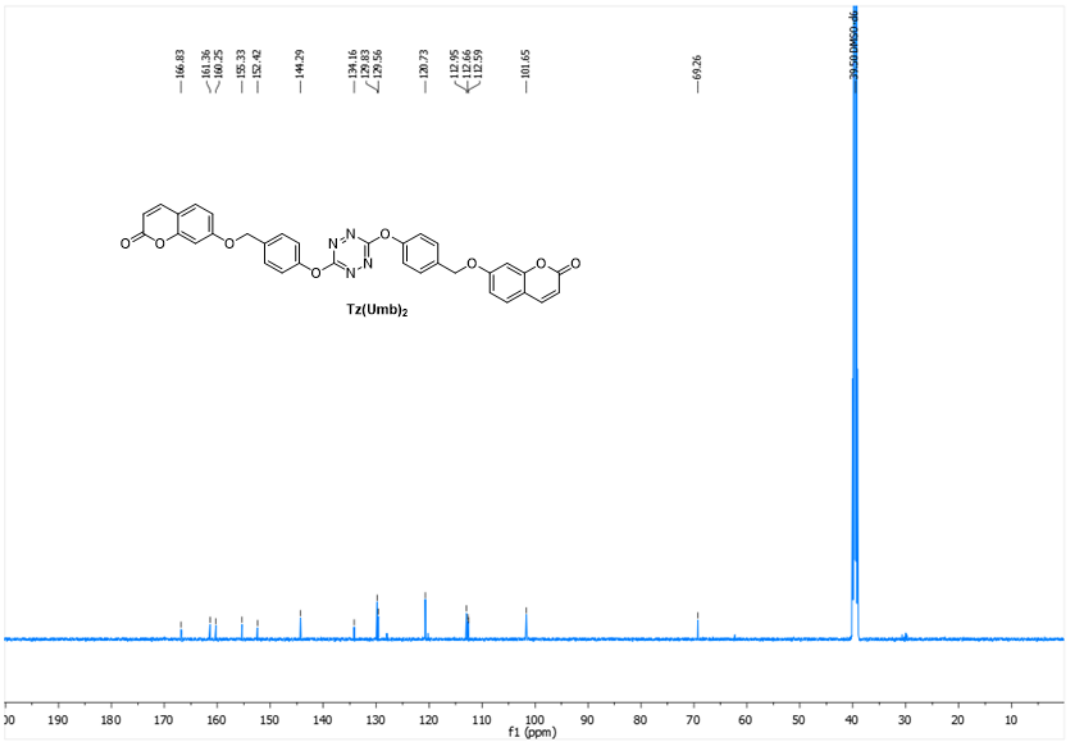


Fig. S10. ^{13}C-NMR spectrum (126 MHz, $CDCl_3$, 298 K) of $Tz(Umb)_2$

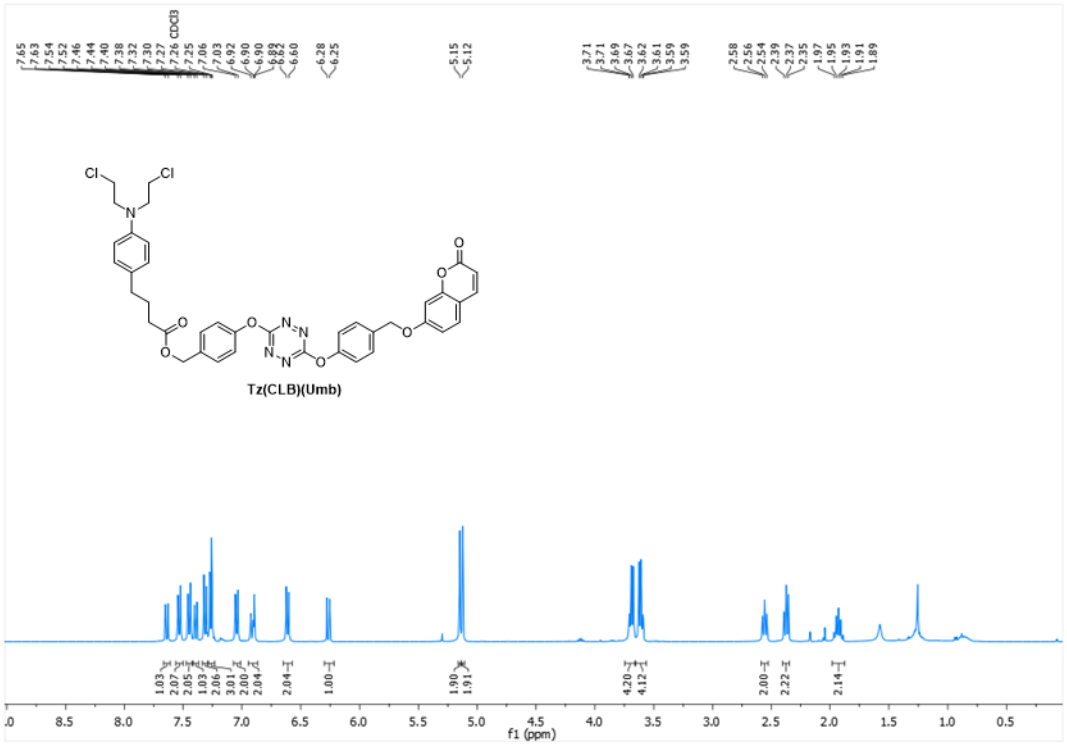


Fig. S11. ^{1}H-NMR spectrum (400 MHz, $CDCl_3$, 298 K) of Tz(CLB)(Umb).

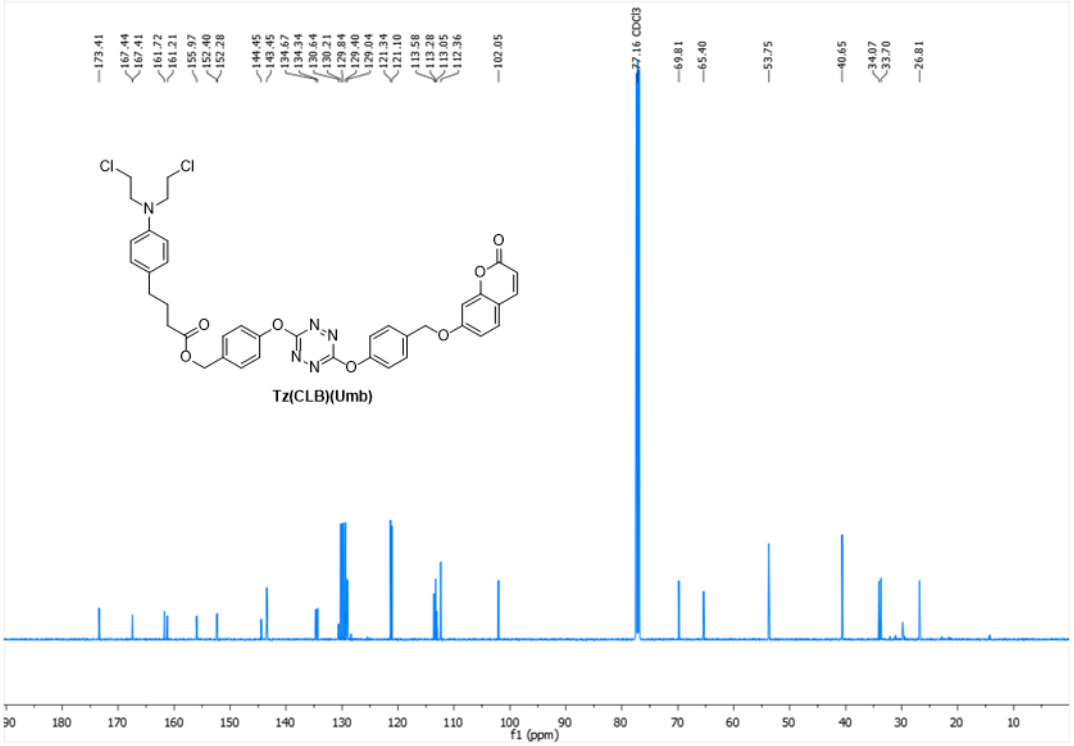


Fig. S12. ^{13}C-NMR spectrum (126 MHz, $CDCl_3$, 298 K) of Tz(CLB)(Umb)

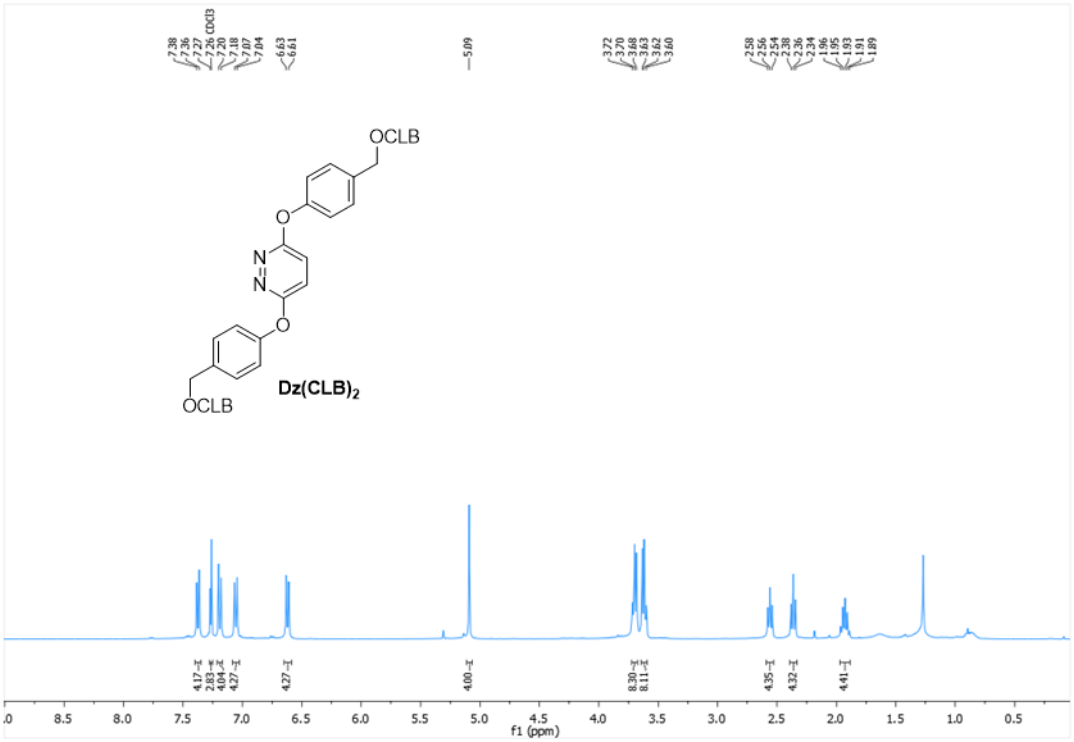


Fig. S13. ^{1}H-NMR spectrum (400 MHz, $CDCl_3$, 298 K) of $Dz(CLB)_2$

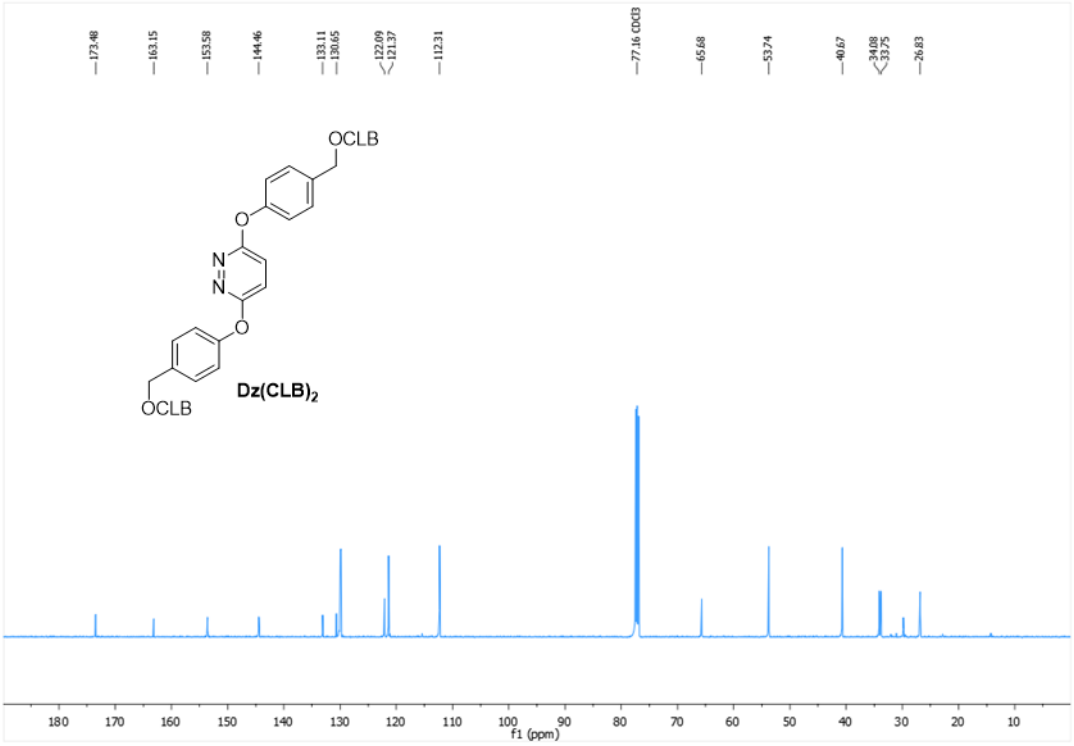


Fig. S14. ^{13}C-NMR spectrum (126 MHz, $CDCl_3$, 298 K) of $Dz(CLB)_2$

3. Absorbance and fluorescence experiments (Supporting figures)

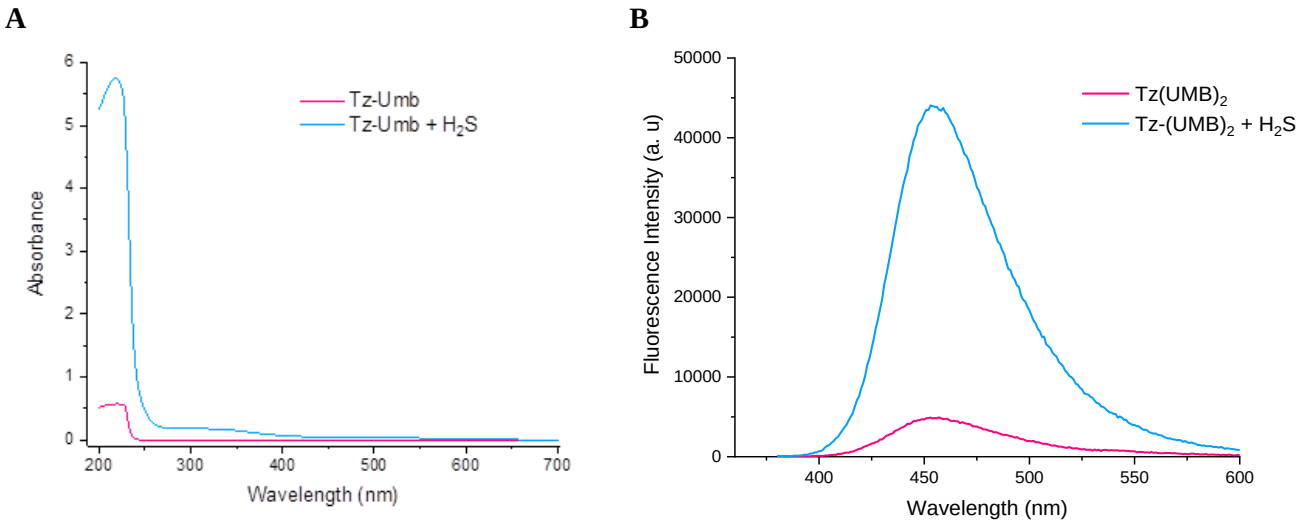


Figura S15 – (**A**) Absorbance spectra of compound **Tz(Umb)$_2$** (100 µM) and (**B**) Fluorescence spectra of compound **Tz-(Umb)$_2$** (10 µM) before and after addition of H_2S (200 µM) in a mixture solution H_2O:DMSO (8%), λ_{ex} = 370 nm.

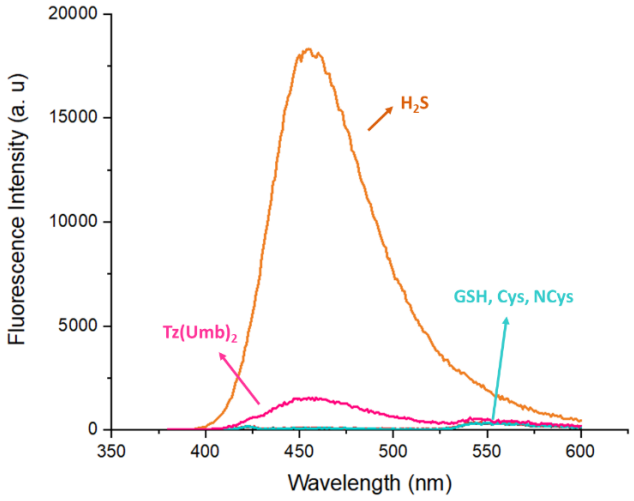


Fig. S16 - Relative fluorescence intensity at λ_{ex}=370 nm of probe **Tz-(Umb)$_2$** (10 µM) with various species (200 µM) at 37ºC in H_2O:DMSO (8%). Gluthatione (GSH, 200 µM), Cysteine (Cys, 200 µM), N-Acetylcysteine (NCys, 200 µM) and H_2S (200 µM).

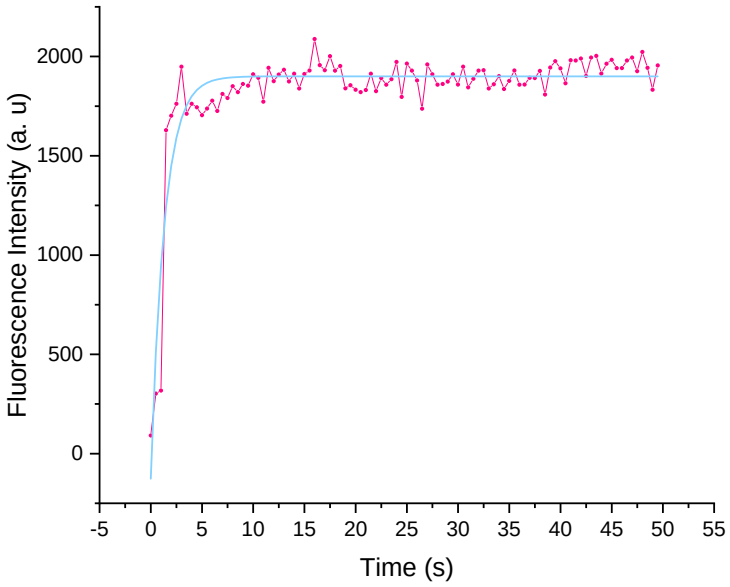


Fig. S17 – Time-course fluorescence intensity at λ_{em}=451 nm of 13 μM probe **Tz-(Umb)$_2$** towards 20 μM H_2S in H_2O:DMSO (8%).

4. ^{1}H NMR experiments

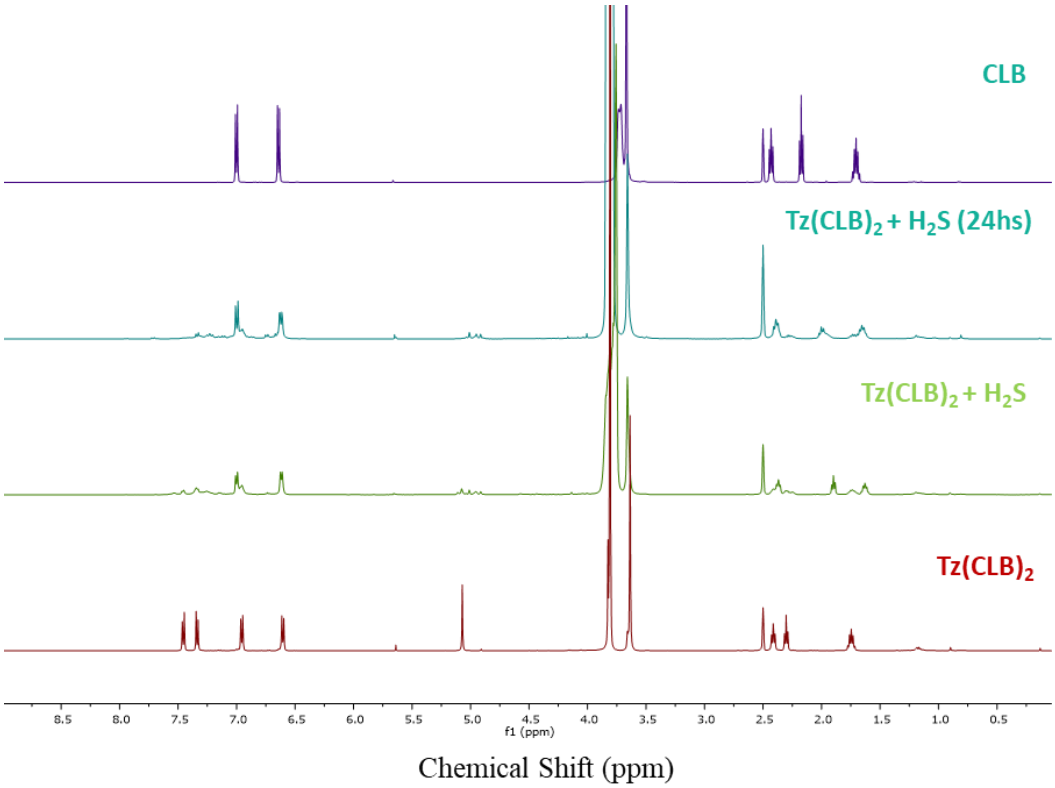


Fig. S18- ^{1}H NMR spectra of Tz(CLB)$_2$ before and after addition of H_2S in DMSO-d_6:D_2O (9:1).

5. *in vitro* experiments

Materials

Reagents and solvents were used as purchased without further purification. All stock solutions of the investigated compounds were prepared by dissolving the powered materials in appropriate amounts of DMSO. The final concentration of DMSO never exceeded 5% (v/v) in biological cytotoxicity assays.

Cytotoxicity assays

Cells were cultured according to the supplier's instructions. Cells were seeded in 96-well plates at a density of 2–2.5 $\times 10^3$ cells per well and incubated overnight in 0.1 mL of media supplied with 10% Fetal Bovine Serum (Lonza) in 5% CO_2 incubator at 37 °C. On day 2, drugs were added and samples were incubated for 48 h. After treatment, 10 μL of cell counting kit-8 was added into each well for additional 2 h incubation at 37ºC. The absorbance of each well was determined by an Automatic Elisa Reader System at 450 nm wavelength.

		Cell line, IC_{50} (μM)		
Entry	Compound	SK-OV3	HCT-116	MRC-5
1	**Tz(CLB)$_2$**	>50	**3.72 ± 1.90**	>50
2	**Dz(CLB)$_2$**	>50	>50	>50
3	**Tz(Umb)$_2$**	>50	19.45 ± 2.18	>50
4	**Tz(CLB)(Umb)**	>50	36.50 ± 2.61	>50
5	**CLB**	>50	>50	>50

Table S1. Antiproliferative activity of **Tz(CLB)$_2$**, **Dz(CLB)$_2$**, **Tz(Umb)$_2$**, **Tz(CLB)(Umb)**, and **CLB**.

Photo-images of cytotoxicity activity of **Tz(CBL)$_2$** against MRC-5, SK-OV3 and HCT-116 cancer cell lines

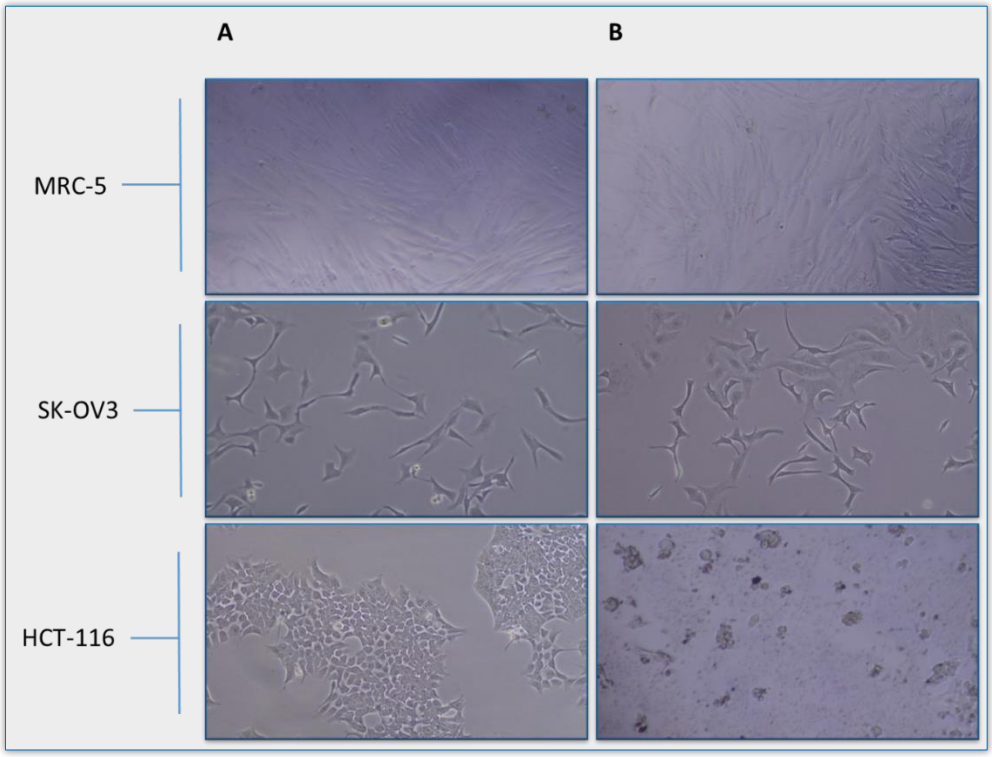


Fig. S19. A) Cells seen under a microscope before adding compound **Tz(CLB)$_2$**. B) Cells seen under the microscope after being incubated 48h in presence of compound **Tz(CLB)$_2$** at a concentration of 50µM.

6. Compound stability

Tz(CLB)$_2$ (0.012 M) was disolved in small amount of DMSO (8%) and was suspended in culture medium (Dulbecco´s Modified Eagle´s Medium – high glucose). After stirring the mixture for 72 hours at room temperature, it was extracted with AcOEt (3 x 2 mL). The organic phases were dried with Na_2SO_4 and the solvent was removed under vacuum. The ^{1}H-RMN showed that the prodrug did not undergo any change.

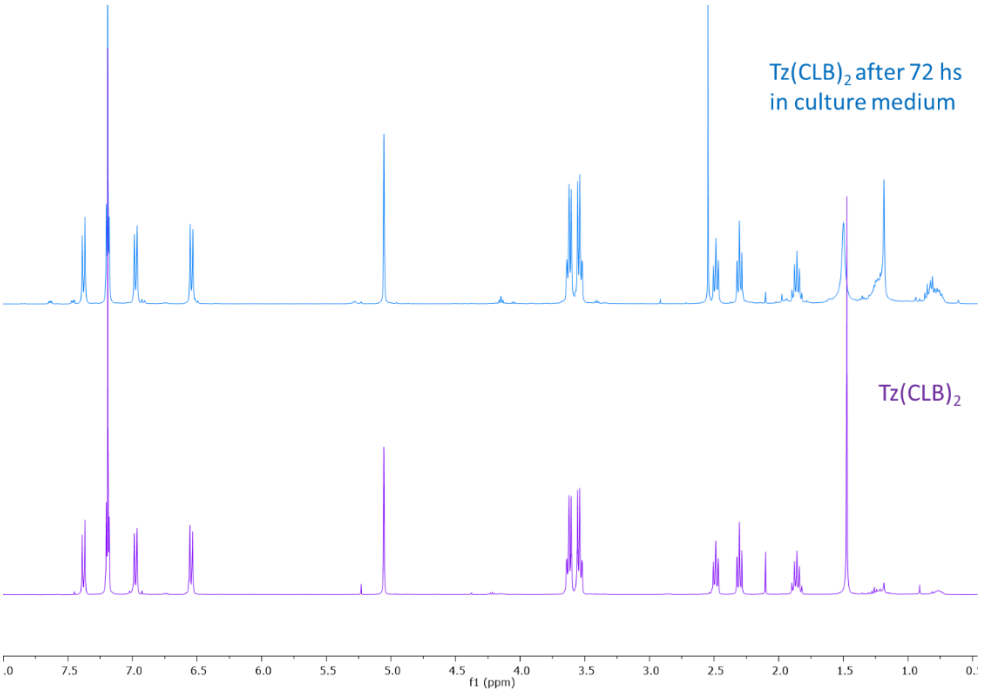


Fig. S20- ^{1}H NMR ($CDCl_3$) spectra of $Tz(CLB)_2$ before and after of 72 hours in culture medium.

7. References

1 - Del Vecchio, A.; Talbot, A.; Caillé, F.; Chevalier, A.; Sallustrau, A.; Loreau, O.; Destro, G.; Taran, F.; Audisio, D. *Chem. Commun*., **2020**,*56*, 11677-11680.

2- Smith, J. M.; Vitali, F.; Archer, S. A.; Fasan, R. *Angew. Chem. Int. Ed*. **2011**,*50*, 5075 –5080.

3 - Santos, T.; Rivero, D. S.; Pérez-Pérez, Y.; Martín-Encinas, E.; Pasán, J.; Hernández Daranas, A.; Carrillo, R. *Angew.Chem.Int.Ed*. **2021**,*60*,18783–18791.