Electronic Supplementary Information

A tetravalent sialic acid-coated tetraphenylethene luminogen with aggregation-induced emission characteristics: design, synthesis and application for sialidase activity assay, high-throughput screening of sialidase inhibitors and diagnosis of bacterial vaginosis

Guang-jian Liu,[a] Beihan Wang,[b] Yuan Zhang,[a] Guo-wen Xing,[a] Xiaoli Yang,[b] and Shu Wang [c]

[a] College of Chemistry, Beijing Normal University, Beijing 100875, China.

[b] Department of Laboratory Medicine, the General Hospital of Chinese People's Armed Police Forces, Beijing, 100039, China

[c] Key Laboratory of Organic Solids, Institute of Chemistry, Chinese Academy of Sciences, Beijing 100190, China

Email: gwxing@bnu.edu.cn; yangxiaolitwins@163.com

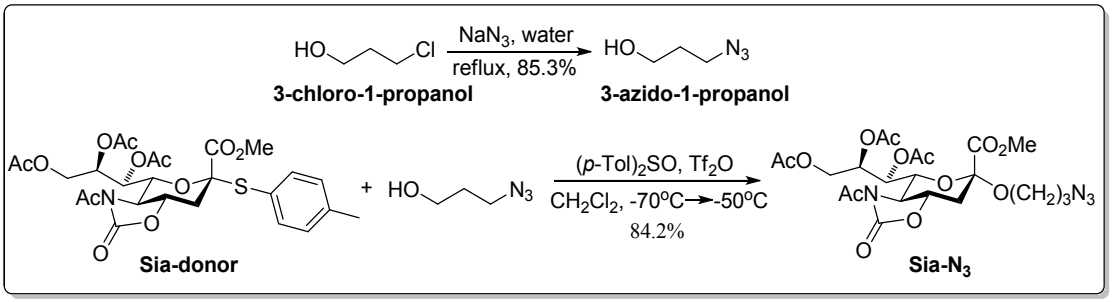
Contents

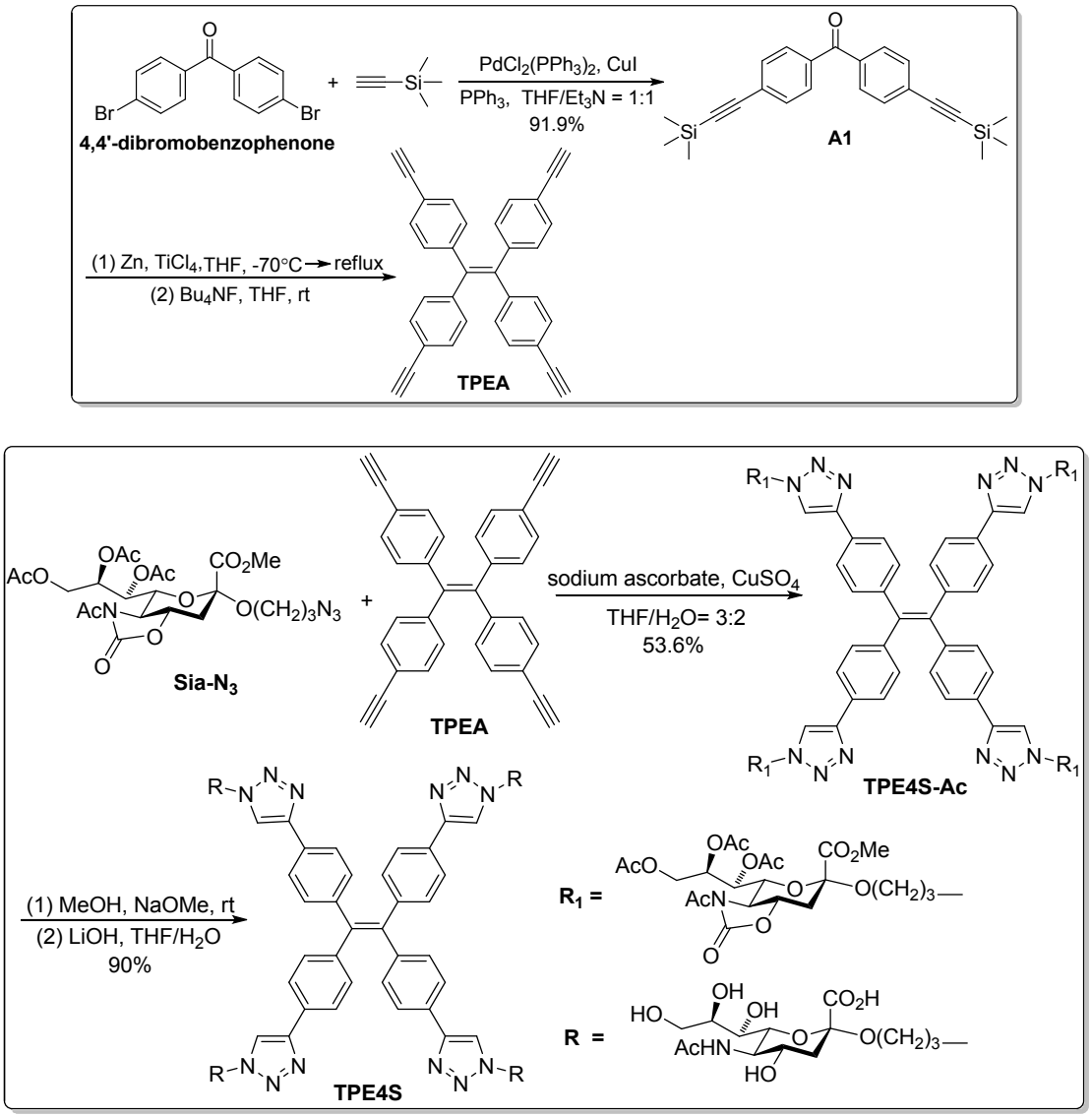
1. Materials and Instruments.

All chemicals were purchased as reagent grade and used without further purification unless otherwise noted below. Sialidase from *Clostridium perfringens* (type V, Sigma product number: N2876, 15.3 U/mg solid) and Sialidase from *Vibrio cholerae* (type III, Sigma product number: N7885, 2 U in buffered aqueous solution) were purchased from Sigma. Chemical reactions were monitored by analytical thin-layer chromatography (TLC) on silica gel F254 glass plates and revealed with UV light (254 nm or 365 nm) or iodine or EtOH-H_2SO_4(4%). Flash column chromatography was performed on silica gel (200-300 mesh) or BioGel P-2 fine resins (Bio-Rad, Hercules, CA).

Nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra were measured at room temperature with a Bruker Avanced III 400 MHz spectrometer or JEOL's NMR (400 or 600 MHz) spectrometer. Chemical shifts are indicated in ppm and coupling constants (J) in Hz. High-resolution electrospray ionization mass spectra (HRMS-ESI) were recorded with a Waters LCT Premier XEmass spectrometer. Particle size data were obtained through dynamic light scattering (ZetaPLUS, Brookhaven Instruments Corp). Fluorescence emission spectra were recorded on a FS5 spectrometer (Edinburgh Instruments) with samples contained in quartz cells. Calorimetric assays were carried out on a VP-ITC calorimeter (MicroCal).

2. Synthesis of probe TPE4S.





Scheme S1. Synthetic routes to probe **TPE4S**.

3-azido-1-propanol. The synthesis was conducted according to the previous literature[1]. Sodium azide (6.5 g, 100 mmol) was dissolved in 30 mL water, then 3-chloro-1-propanol (4.727 g, 50 mmol) was added. The mixture was stirred at 80°C for 30 h then allowed to reach room temperature. The product was extracted with methylene chloride twice, dried over anhydrous sodium sulfate, filtrated and concentrated as colorless oil (4.2672 g, 85.3%, R_f = 0.48, petroleum : EtOAc = 7:3). ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.77 (t, J = 5.9 Hz, 2H), 3.46 (t, J = 6.6 Hz, 2H), 1.88 – 1.81 (m, 2H). The spectroscopic data coincide with the previous report[1].

Sia-donor. The synthesis was conducted according to the previous literature[2]. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43 (d, *J* = 8.0 Hz, 2H), 7.15 (d, *J* = 7.9 Hz, 2H), 5.60 – 5.50 (m, 1H), 5.35 (td, *J* = 6.4, 2.8 Hz, 1H), 4.44 (dd, *J* = 12.2, 2.8 Hz, 1H), 4.34 (d, *J* = 9.4 Hz, 1H), 4.21 (dd, *J* = 12.2, 6.7 Hz, 1H), 3.95 (td, *J* = 12.6, 3.6 Hz, 1H), 3.69 – 3.51 (m, 4H), 3.08 (dd, *J* = 12.1, 3.6 Hz, 1H), 2.46 (s, 3H), 2.37 (s, 3H), 2.17 (s, 3H), 2.13 – 2.04 (m, 7H). The spectroscopic data coincide with the previous report[2a].

Sia-N_3. This compound was synthesized according to the previously published procedure[3]. To a mixture of **Sia-donor** (144. 5 mg, 0.248 mmol), (*p*-Tol)$_2$SO (114.5 mg, 0.497 mmol) and activated 3Å powdered sieves in flame-dried glass vessel was added anhydrous dichloromethane (CH_2Cl_2, 8 mL), which was freshly distilled over calcium hydride. The resultant mixture was stirred at -70°C for 15 min, followed by addition of trifluoromethanesulfonic anhydride (50 μL). After activation for 30 min, a solution of 3-azido-1-propanol (37.7 mg, 0.373 mmol) in anhydrous dichloromethane (2 mL) was added, and the reaction mixture was stirred at -70°C for 2 h and then stirred at -50°C for another 2 h. The reaction was quenched with Et_3N (0.2 mL), diluted with CH_2Cl_2, filtered through Celite, washed with saturated brine and dried over anhydrous $MgSO_4$. After filtration and evaporation, the residue was purified by column chromatography to give the desired product **Sia-N_3** (116.8 mg, 84.2%, R_f = 0.55, petroleum : EtOAc = 2:3). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.56 (dd, *J* = 8.2, 1.3 Hz, 1H), 5.44 – 5.37 (m, 1H), 4.60 (dd, *J* = 9.4, 1.2 Hz, 1H), 4.35 (dd, *J* = 12.3, 2.8 Hz, 1H), 4.06 – 3.94 (m, 2H), 3.86 – 3.76 (m, 4H), 3.68 (dd, *J* = 11.0, 9.6 Hz, 1H), 3.39 – 3.35 (m, 3H), 2.82 (dd, *J* = 12.1, 3.5 Hz, 1H), 2.45 (s, 3H), 2.10 – 2.05 (m, 7H), 2.00 (s, 3H), 1.86 – 1.76 (m, 2H).

A1 and **TPEA** were synthesized according to previously published methods[4] and the ^{1}H NMR data of **A1** and **TPEA** were as follows. **A1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (d, *J* = 8.4 Hz, 4H), 7.56 (d, *J* = 8.4 Hz, 4H), 0.27 (s, 18H). **TPEA.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.25 (d, *J* = 8.2 Hz, 8H), 6.94 (d, *J* = 8.3 Hz, 8H), 3.06 (s, 4H).

TPE4S-Ac. A solution of sodium ascorbate (8.5 mg) in water (1 mL) and a solution of $CuSO_4 \cdot 5H_2O$ (5.4 mg) in water (1 mL) were added to a solution of **Sia-N_3** (119.7 mg, 0.214 mmol) and **TPEA** (18.3 mg, 0.0427 mmol) in THF (6 mL) under an Ar atmosphere and the mixture was stirred vigorously at 60°C. After ca. 4 h, additional

sodium ascorbate (8.5 mg in 1 mL water) and $CuSO_4 \cdot 5H_2O$ (5.4 mg in 1 mL water) were added. The reaction was continued at 60°C under argon atmosphere for 32 h. After cooled to room temperature, the reaction mixtures were diluted with aqueous solution of NH_4Cl and extracted with CH_2Cl_2. The organic phase was dried over anhydrous sodium sulfate, filtered and concentrated. The crude product was purified by flash chromatography affording pure **TPE4S-Ac** (61.0 mg, 53.6%, R_f = 0.57, EtOAc: methanol = 30:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (s, 1H), 7.58 (d, *J* = 8.2 Hz, 2H), 7.13 (d, *J* = 8.2 Hz, 2H), 5.56 (dd, *J* = 8.4, 1.3 Hz, 1H), 5.41 (td, *J* = 7.9, 2.7 Hz, 1H), 4.61 (dd, *J* = 9.4, 1.3 Hz, 1H), 4.57 – 4.41 (m, 2H), 4.36 (dd, *J* = 12.2, 2.7 Hz, 1H), 4.05 – 3.93 (m, 2H), 3.91 – 3.81 (m, 1H), 3.77 – 3.66 (m, 4H), 3.44 – 3.30 (m, 1H), 2.83 (dd, *J* = 12.1, 3.4 Hz, 1H), 2.48 (s, 3H), 2.27 – 2.15 (m, 2H), 2.13 – 2.02 (m, 7H), 1.99 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 172.14, 170.87, 170.35, 170.15, 168.78, 153.73, 147.49, 143.44, 140.74, 132.06, 129.15, 125.27, 120.23, 99.16, 75.62, 74.91, 71.82, 68.84, 63.43, 62.44, 59.13, 53.23, 47.37, 36.58, 30.53, 24.78, 21.23, 21.03, 20.88. HRMS calcd for $C_{122}H_{140}N_{16}O_{52}Na$ $[M + Na]^+$: 2683.8695, found: 2683.9348.

TPE4S. A solution of NaOMe in MeOH (5.4 M, 0.1 mL) was added to a solution of **TPE4S-Ac** (60 mg, 0.0225 mmol) in methanol (3 mL) and the reaction was stirred at room temperature for 3 h. After neutralization with Amberlite IR-120 (H^+ form), filtration and evaporation of solvents, the partially deprotected compound was dissolved in 3 mL of a 2:1 THF-H_2O mixture. To this solution was added $LiOH \cdot H_2O$ (26 mg) and the reaction was stirred at room temperature overnight. Then Amberlite IR-120 (H^+ form) was added to neutralize the reaction mixture. After filtration, the solvent was removed by a rotary evaporator and the crude product was purified using size exclusion chromatography (eluent system: 50 mM ammonium formate aqueous solution). Product-containing fractions were collected, concentrated and freeze-dried to afford the probe **TPE4S** (40.5 mg, 90%). ^{1}H NMR (400 MHz, D_2O) δ 7.98 (s, 1H), 7.44 (s, 2H), 6.96 (s, 2H), 4.55 – 4.15 (m, 2H), 3.84 – 3.33 (m, 10H), 2.67 – 2.49 (m, 1H), 2.01 (s, 1H), 1.94 (s, 3H), 1.58 – 1.42 (m, 1H). ^{13}C NMR (101 MHz, D_2O) δ 175.61, 174.08, 147.25, 144.16, 132.59, 128.53, 125.67, 100.99, 73.26, 72.25, 68.82, 68.69, 62.96, 61.74, 52.68, 48.41, 40.93, 30.29, 22.69. HRMS calcd for $C_{90}H_{117}N_{16}O_{36}$ $[M + H]^+$: 1998.7844, found: 1998.7851.

3. General procedure for fluorescence spectra measurements.

Fluorescence titration experiments. 6 μL stock solution of TPE4S (10 mM in water) was added to different concentrations of sialidase in phosphate buffered saline (pH 7.1) and the final volume of the sensing system was 3 mL. The fluorescence emission spectra of the mixtures were collected after shaking at 37°C for 1 h, 2h or 24 h.

Fluorescence responses to sialidase from *Clostridium perfringens* at different pH values. PBS buffer solutions with different pH values (4.74-7.95) were prepared beforehand. Then 6 μL stock solution of TPE4S (10 mM in water) and 60 μL stock solution of sialidase (1 mU/mL) were added into 2934 μL of PBS solutions with different pH values. The control groups were set to be TPE4S (20 μM) only in different PBS. Fluorescence emission spectrum of each mixture was measured after shaking at 37°C for 1 h.

Selectivity of TPE4S. Aqueous stock solution of TPE4S (5 mM) and stock solutions of sugars (10 mM) were added into phosphate buffer (pH 7.1) and the final concentrations of TPE4S and sugar were 10 μM and 200 μM, respectively. Control group contained TPE4S and sialidase, and blank group contained 10μM TPE4S only in PBS solution. Fluorescence emission spectra were measured after aging for 1 h. In the case of metal ions, 1 mL 20 μM TPE4S in HEPES buffer (10 mM, pH 7.1) and 1 mL 40 μM common metal ions in HEPES buffer were mixed and the blank group without common metal ions contained an equal quantity of TPE4S in HEPES buffer. The mixtures were shaken for 1 h at 37°C before fluorescence measurements.

4. Measurements of Michaelis constants (K_M).

The Michaelis-Menten based kinetic data were obtained using the powerful and convenient isothermal titration calorimetric (ITC) technique, which can be ubiquitously applied to enzyme-catalyzed reaction with no prior chemical modifications of any participates. Here, we presented the ITC assay on the sialidase from *Clostridium perfringens* and substrate TPE4S. Solutions were degassed by means of a vacuum degasser and thermostated prior to any experimental run.

Firstly, the apparent molar enthalpy (ΔH_{app}) of hydrolysis of TPE4S catalyzed by sialidase was determined by ITC as follows. The calorimeter sample cell (1.43 mL) was filled with PBS buffer containing 150 mU/mL sialidase. The syringe was loaded with 1.5

mM TPE4S in the same buffer as for the one in the sample cell and titrated by three injections of 4 μL. The interval between injections was 40 min to ensure that all of the substrate injected each time was converted to product completely. The stirring speed was maintained at 307 rpm. In addition, the heat generated by dilution of the substrate was determined under the same experimental conditions except for the absence of sialidase. The molar enthalpy (ΔH_{app}) was then accurately determined by subtracting the heat of dilution from the first total heat evolved.

Then we designed the enzyme kinetic investigations to be carried out by using the so-called multiple injection method. The sample cell was filled with 15 mU/mL sialidase solutions. The syringe was loaded with 1.5 mM TPE4S in the same buffer and titrated by 30 injections of 2 μL each. The interval between each injection was 180 s. The data obtained were analyzed by inputting ΔH_{app} in enzyme assay mode in the Origin software and the kinetic parameter K_M was obtained when fitting the data points in Figure 2c.

Furthermore, using the similar procedure described above, the ITC assay on the sialidase from *Vibrio cholerae* and substrate TPE4S was also performed.

5. Screening of sialidase inhibitors.

100 μL of different concentrations (0.015, 0.03, 0.06, 0.12, 0.25, 0.5, 1, and 2 mM in PBS) of potential sialidase inhibitors (Neu5Ac2en, zanamivir, oseltamivir acid and oseltamivir) were set on a 96-well plate (Thermo Scientific, light-tight, flat-bottom) loaded with 80 μL of 25 mU/mL sialidase from *Clostridium perfringens* and incubated for 30 min. Then 20 μL of 200 μM TPE4S in PBS was added and further incubation at 37°C for 1 h. The control groups were set to be that without inhibitors and the blank groups contained TPE4S buffer solution only. Fluorescence intensity was read (excitation wavelength of 380 nm and emission wavelength of 510 nm) on a microplate reader (Thermo Scientific, Varioskan Flash). The inhibition efficiency of inhibitors was then expressed using the relative fluorescence intensity $(I_C\text{-}I)/(I_C\text{-}I_0)$, where I and I_C are the fluorescence intensity at 510 nm of mixtures with and without inhibitors respectively and I_0 is the fluorescence background of 20 μM TPE4S itself in PBS. Three independent experiments were performed for the calculation of the IC_{50} values.

6. Diagnosis of bacterial vaginosis (BV).

Reproductive-aged women (20 – 60 years) presented in gynaecological clinics in the General Hospital of Chinese People's Armed Police Forces were recruited into the study. Two vaginal swabs were collected aseptically from each patient. One swab was used for preparation of a slide for vaginal cleanliness determination and the second swab was put into physiological saline and gently swirled to mix properly for BVBlue test and TPE4S fluorescence test. BVBlue test was performed using vaginitis detection kit by LTS-V400 vaginitis detector (Zhuhai Lituo Biotechnology Co., Ltd, China). The healthy group and BV group determined by BVBlue test and microscopic observations were then subjected to TPE4S fluorescence test by incubating 150 μL of physiological saline containing vaginal fluid with 7 μL of TPE4S stock solutions (420 μM TPE4S in PBS) in 96-well plate at 37°C for 1 h. After the corresponding sialidase activity was determined fluorescently at 510 nm with an excitation wavelength of 380 nm on a microplate reader, we grade the samples as normal (grade 1, $0< I/I_0 \leq 5$), sialidase weak positive (grade 2, $5< I/I_0 \leq 10$), and sialidase positive (grade 3, $I/I_0 > 10$). I_0 is the fluorescence background of same volume of 20 μM TPE4S only. The Kappa coefficient was used as a measure of reliability of our method and was classified as follows: < 0.20, poor agreement; 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, good agreement; 0.81–1.00, excellent agreement.

7. Supplementary figures.

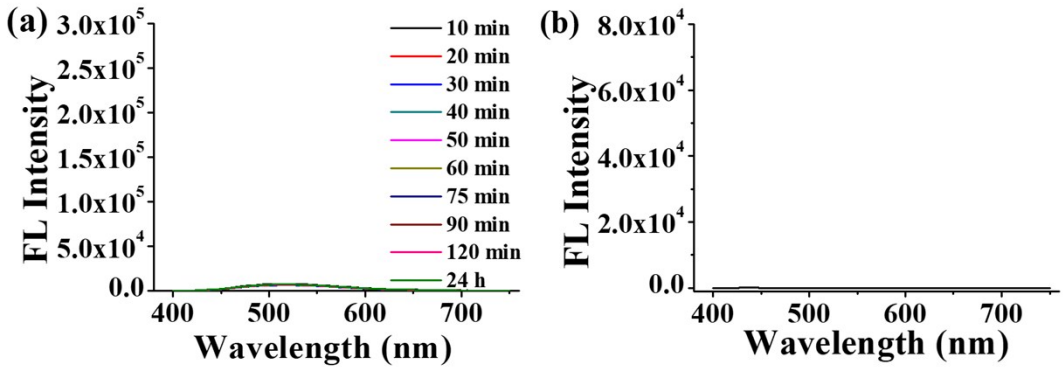


Figure S1. (a) Fluorescence spectra of 20 μM TPE4S in PBS (pH 7.1, 6.7 mM) obtained at different time. (b) FL spectra of 50 mU/mL sialidase from *Clostridium perfringens* in PBS (pH 7.1, 6.7 mM). λ_{ex} = 380 nm.

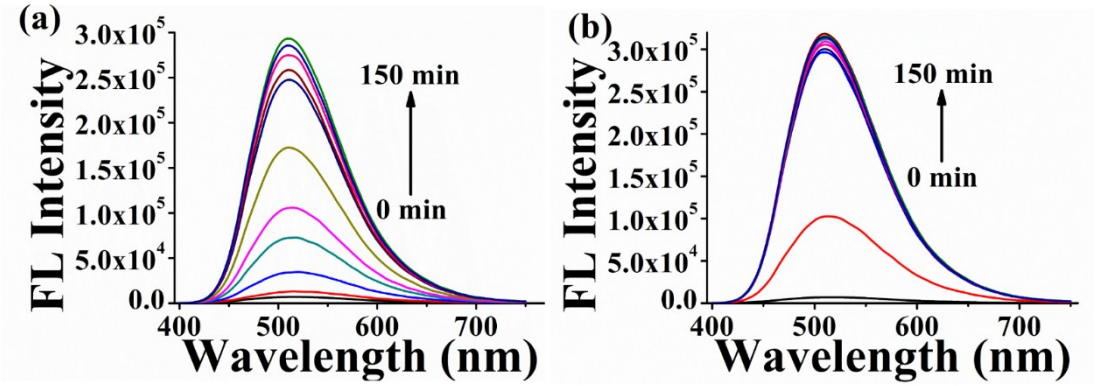


Figure S2. Fluorescence response of 20 μM TPE4S to (a) 10 mU/mL and (b) 50 mU/mL sialidase from *Clostridium perfringens*. Spectra were acquired before and 10, 20, 30, 40, 50, 60, 75, 90, 120, and 150 min after sialidase was added. λ_{ex} = 380 nm.

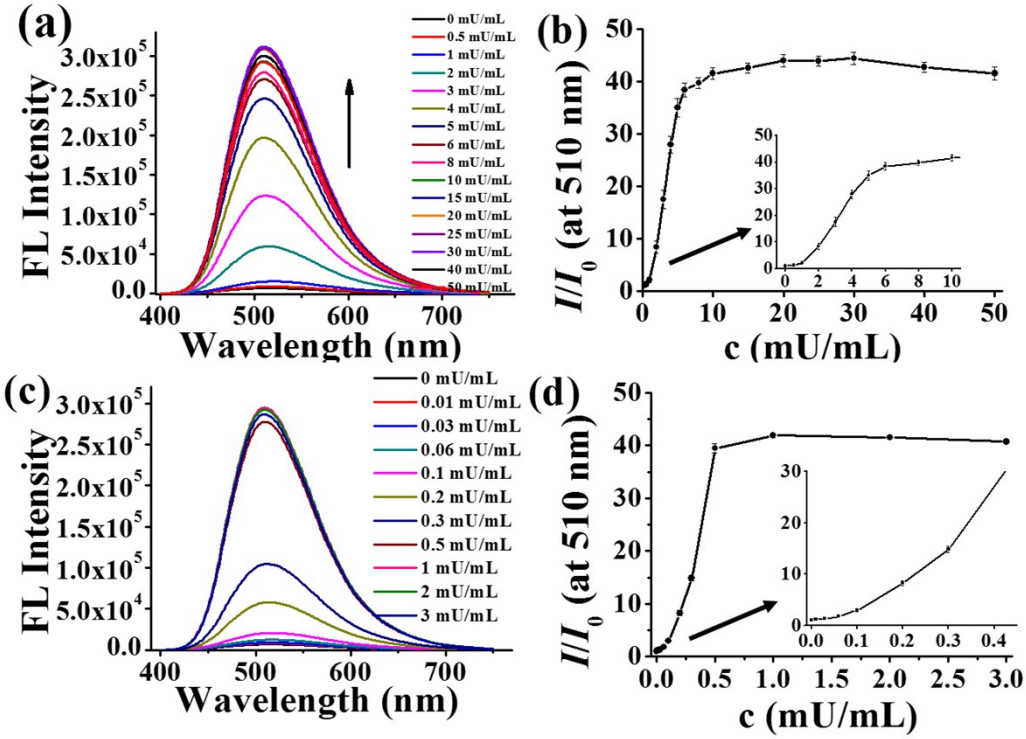


Figure S3. (a) Emission spectra and (b) fluorescence responses of 20 μM TPE4S to various concentrations of added sialidase from *Clostridium perfringens*; The spectra were obtained in PBS (pH 7.1, 6.7 mM) 2 h after the addition of sialidase. (c) Emission spectra and (d) fluorescence responses of 20 μM TPE4S to various concentrations of added sialidase from *Clostridium perfringens*; The spectra were obtained in PBS (pH 7.1, 6.7 mM) 24 h after the addition of sialidase. λ_{ex} = 380 nm.

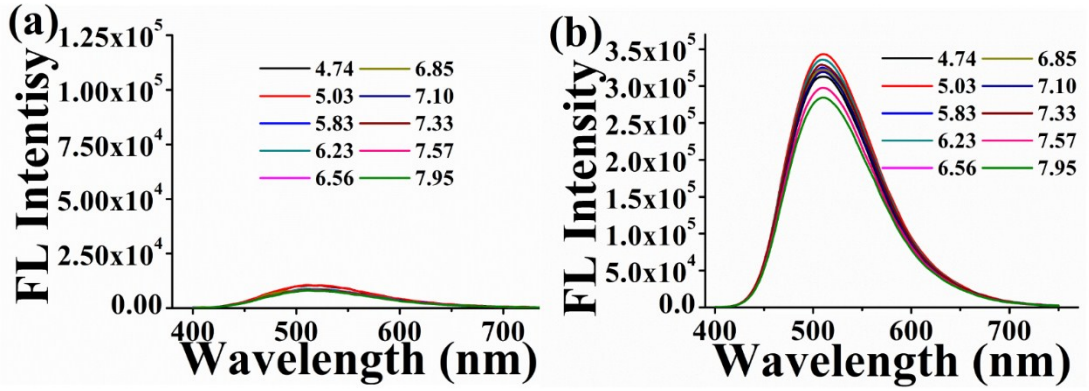


Figure S4. Fluorescence emission spectra of (a) TPE4S (20 μM) and (b) its hydrolytic product at different pH values. λ_{ex} = 380 nm.

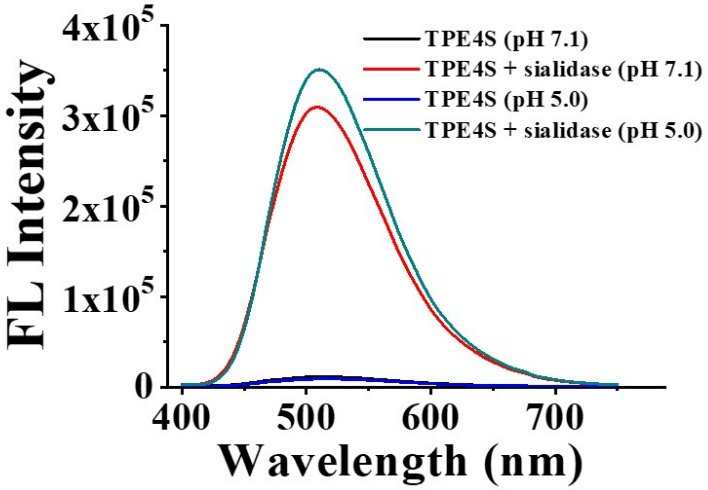


Figure S5. Fluorescence spectra of TPE4S with and without sialidase (50 mU·mL^{-1}) from *Vibrio cholerae* for 1 h in PBS buffer (pH 7.1, 6.7 mM) containing 4 mM $CaCl_2$ or in sodium acetate buffer (pH 5.0) containing 4 mM $CaCl_2$.

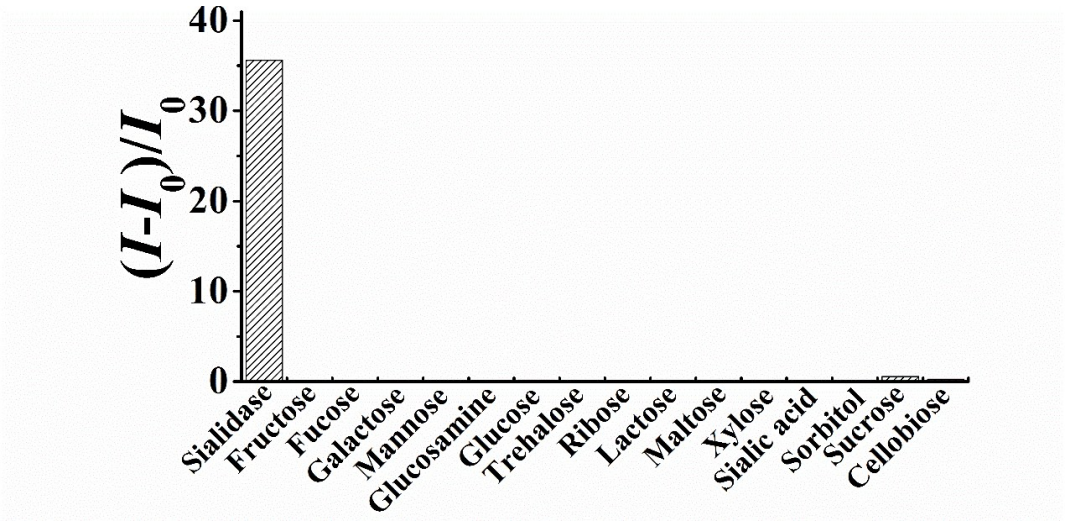


Figure S6. Fluorescence responses of TPE4S (10 μM) to various species [sialidase (50 mU/mL), polyhydroxy compounds (0.2 mM)] in PBS buffer solution (pH 7.1, 6.7 mM) with $\lambda_{ex/em}$ = 380/510 nm, where I_0 and I are the fluorescence intensities of 10 μM TPE4S alone and that upon incubation with different polyhydroxy compounds, respectively

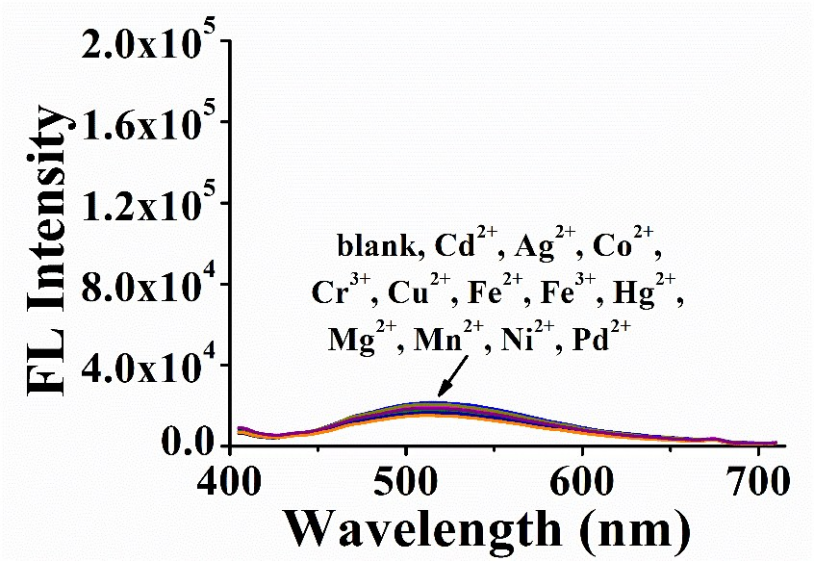
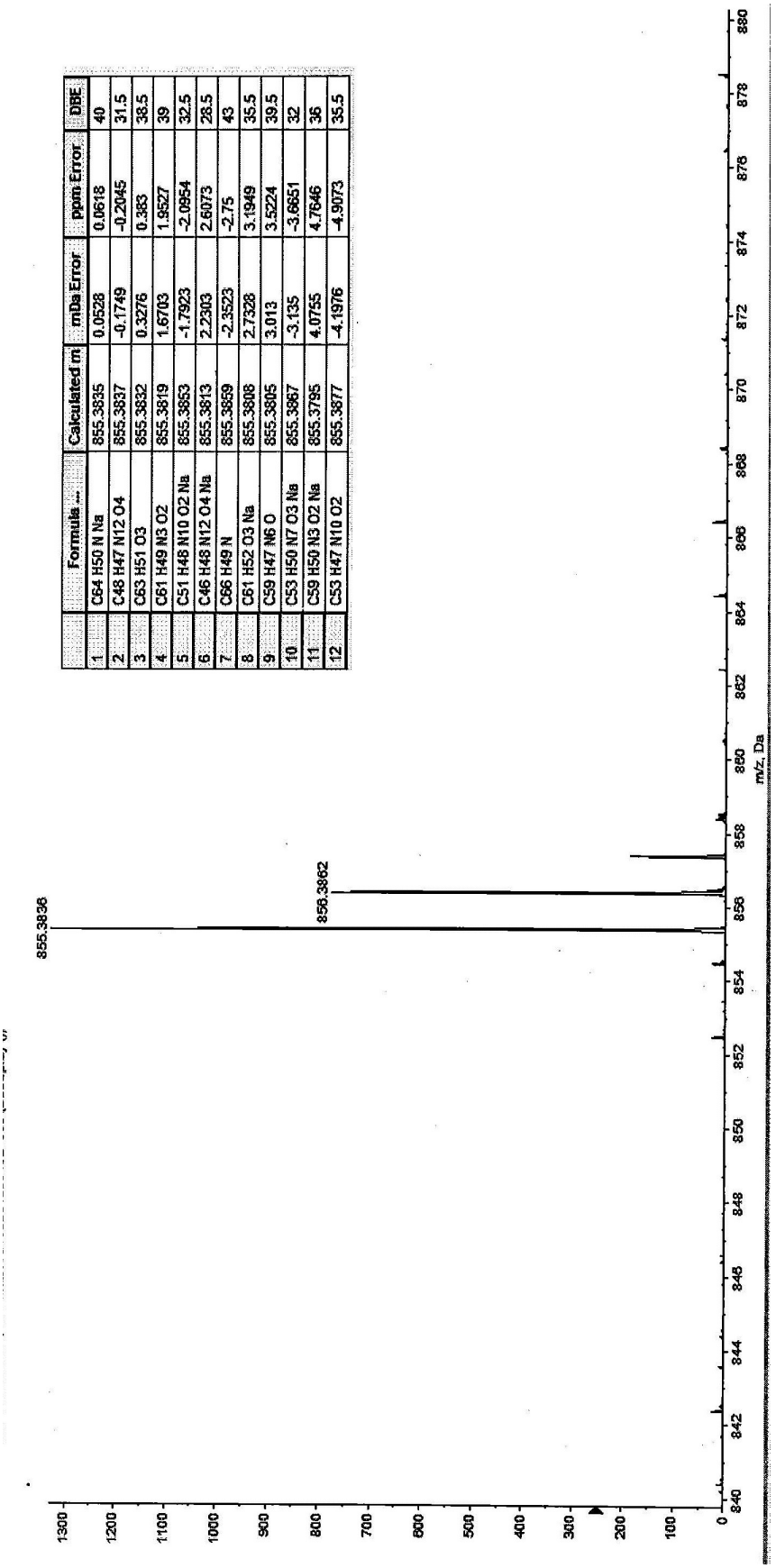


Figure S7. Fluorescence spectra of TPE4S (10 μM) in the presence of various metal ions (2.0 equiv.) in HEPES buffer solution (10 mM , pH 7.1). The spectra were collected with excitation at 380 nm.



	Formula ...	Calculated m	mDa Error	ppm Error	DBE
1	C64 H50 N Na	855.3835	0.0528	0.0618	40
2	C48 H47 N12 O4	855.3837	-0.1749	-0.2045	31.5
3	C63 H51 O3	855.3832	0.3276	0.383	38.5
4	C61 H49 N3 O2	855.3819	1.6703	1.9527	39
5	C51 H48 N10 O2 Na	855.3853	-1.7923	-2.0954	32.5
6	C46 H48 N12 O4 Na	855.3813	2.2303	2.6073	28.5
7	C66 H49 N	855.3859	-2.3523	-2.75	43
8	C61 H52 O3 Na	855.3808	2.7328	3.1949	35.5
9	C59 H47 N6 O	855.3805	3.013	3.5224	39.5
10	C53 H50 N7 O3 Na	855.3867	-3.135	-3.6651	32
11	C59 H50 N3 O2 Na	855.3795	4.0755	4.7646	36
12	C53 H47 N10 O2	855.3877	-4.1976	-4.9073	35.5

Figure S8. ESI-MS spectra characterization of the hydrolytic product of TPE4S catalyzed by adequate sialidase.

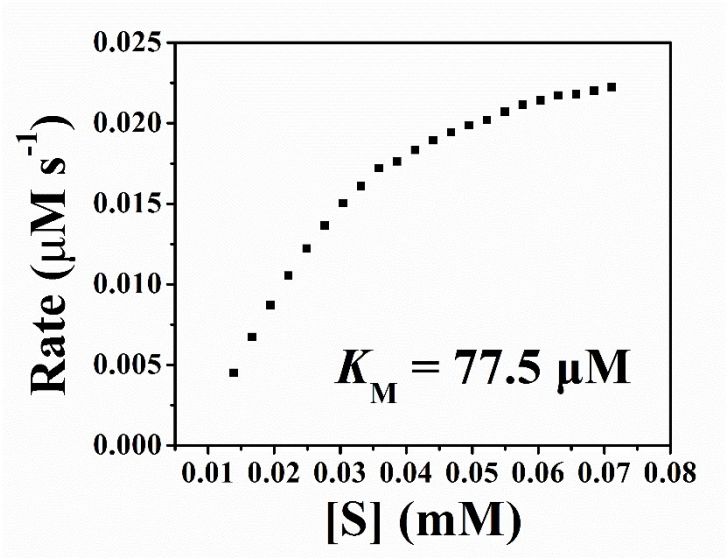


Figure S9. Calorimetric determination of enzyme kinetic parameters for the hydrolysis of TPE4S by sialidase from *Vibrio cholerae* at 37°C.

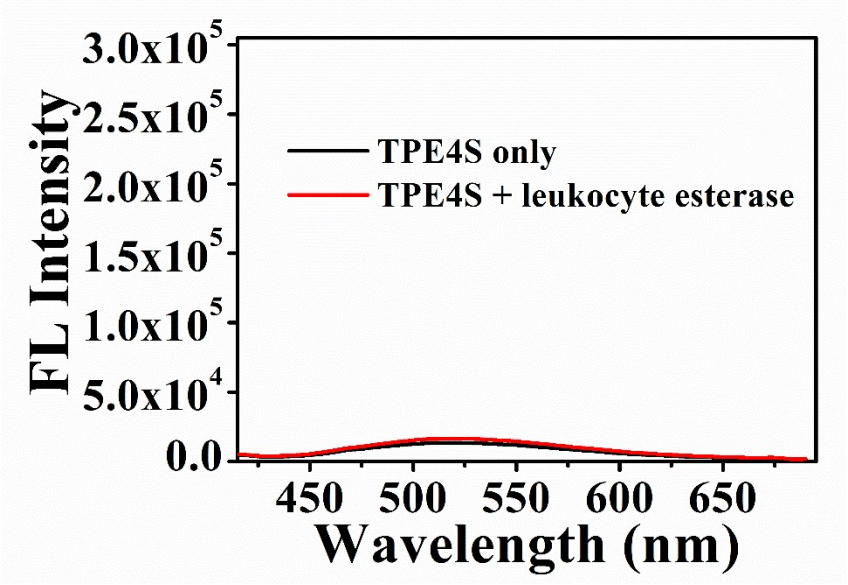


Figure S10. Fluorescence spectra of TPE4S (20 μM) in the absence and presence of leukocyte esterase. The spectra were collected with excitation at 380 nm.

Figure S11. [1]H NMR spectrum of compound **3-azido-1-propanol.**

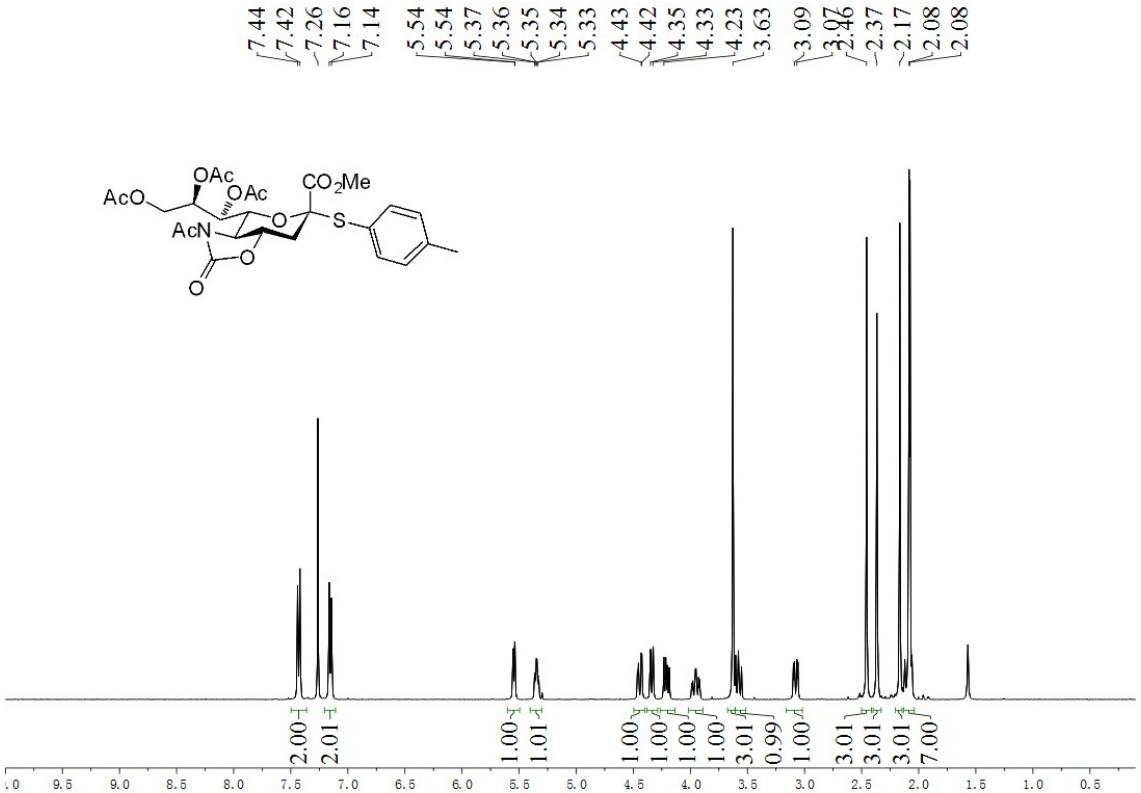


Figure S12. [1]H NMR spectrum of compound **Sia-donor.**

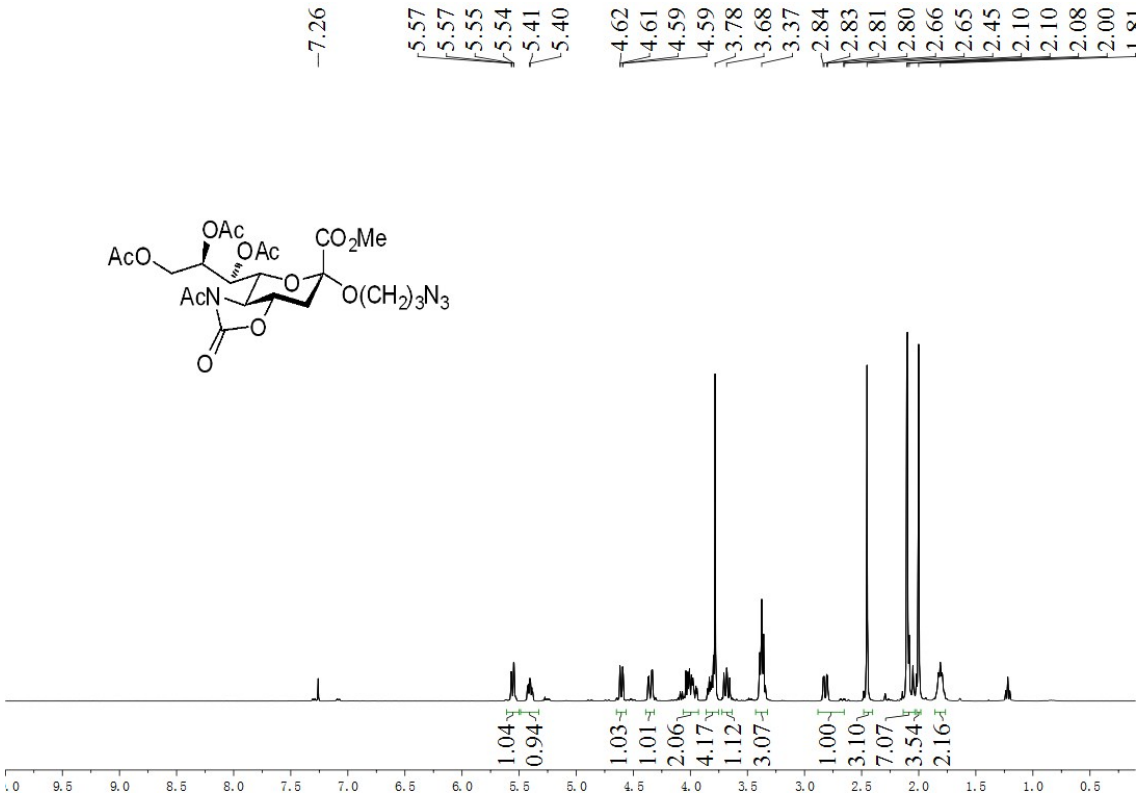


Figure S13. ^{1}H NMR spectrum of compound **Sia-N_3.**

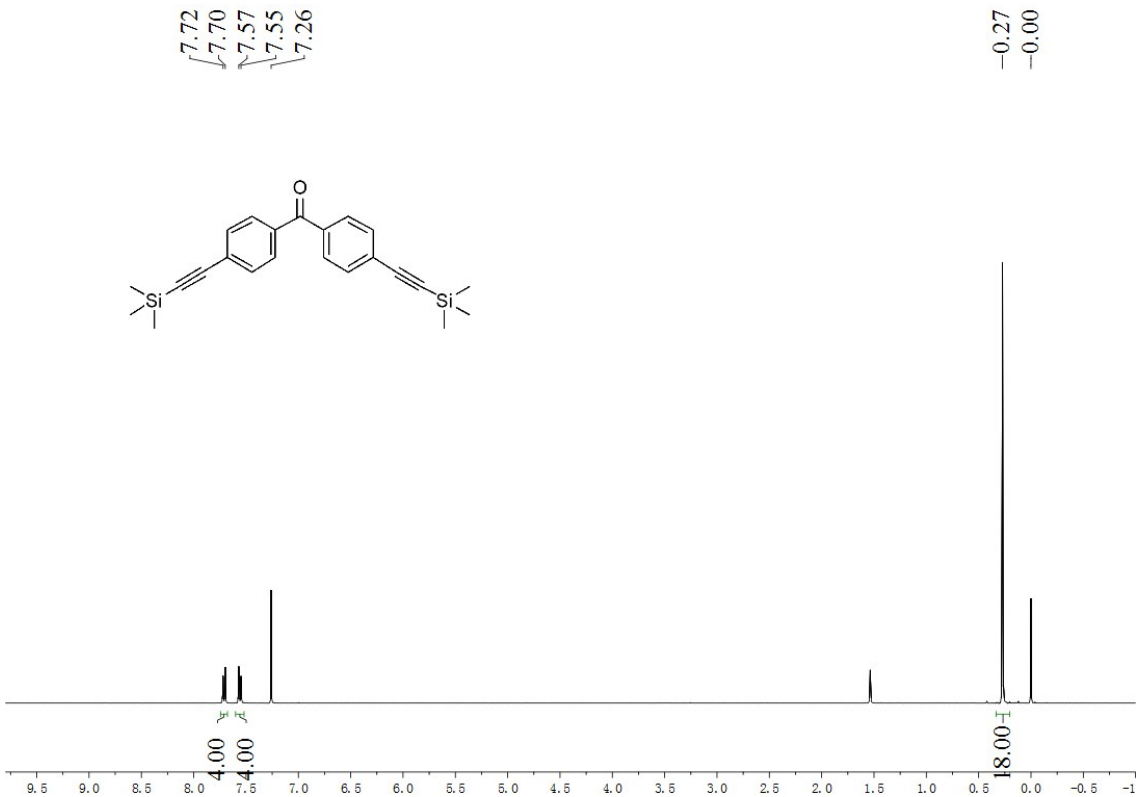


Figure S14. ^{1}H NMR spectrum of compound **A1.**

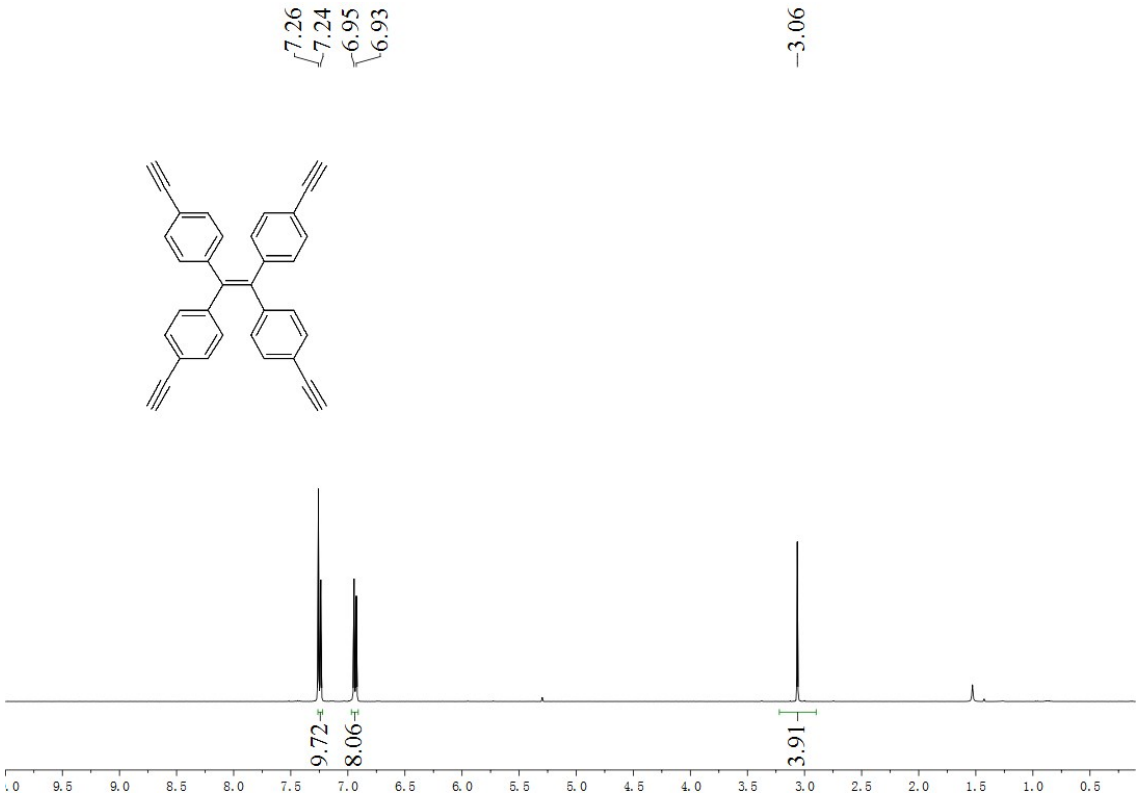


Figure S15. ^{1}H NMR spectrum of compound **TPEA.**

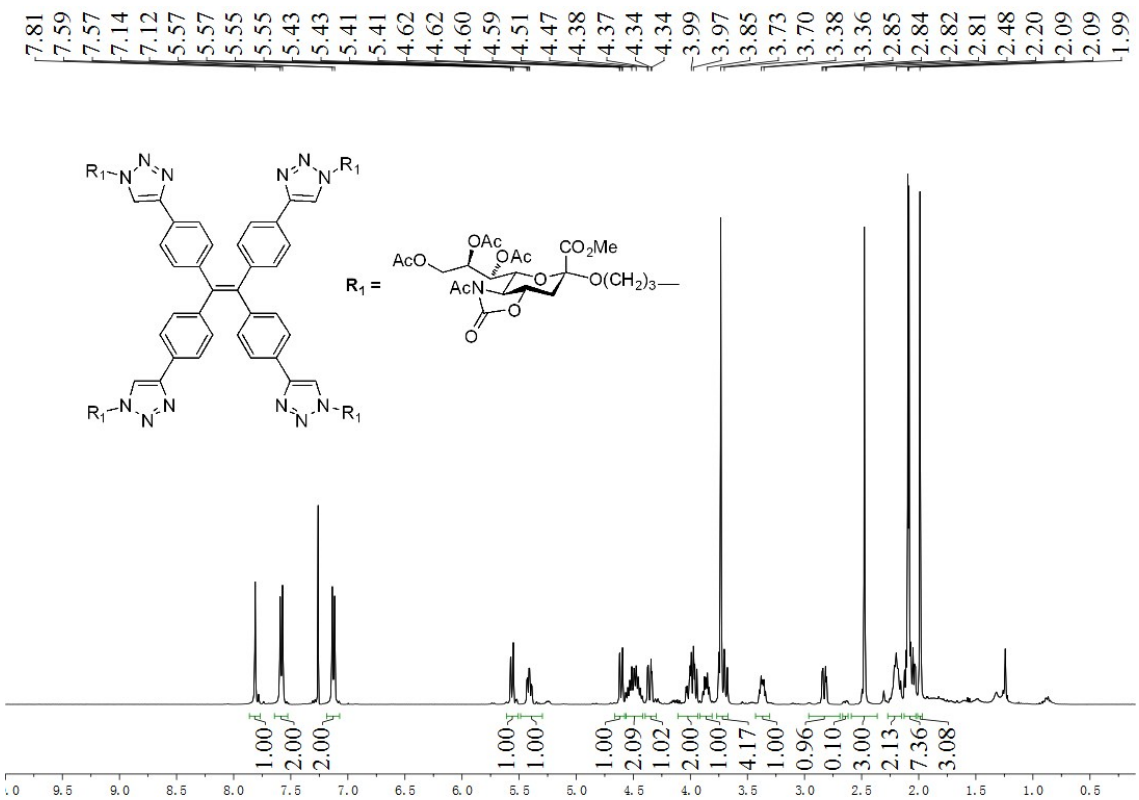


Figure S16. ^{1}H NMR spectrum of compound **TPE4S-Ac** (The normalization of the integrations appear to be for a single arm of the compound).

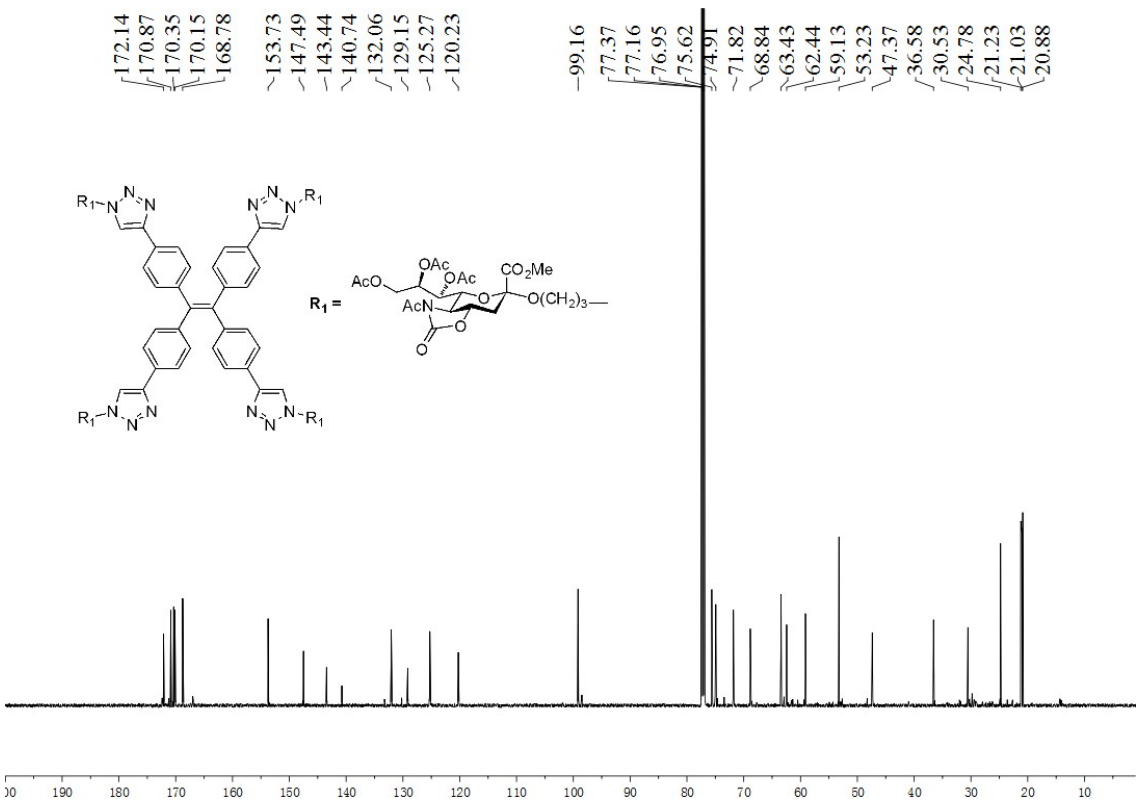


Figure S17. ^{13}C NMR spectrum of compound **TPE4S-Ac.**

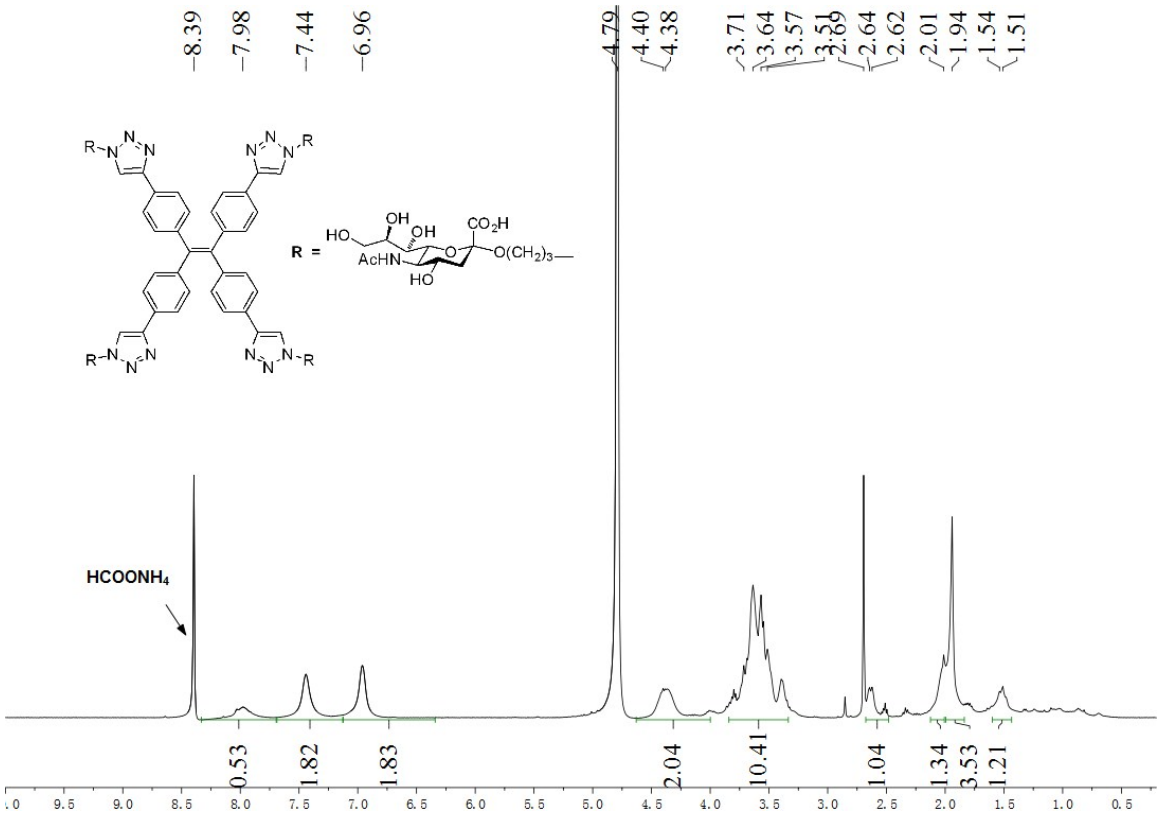


Figure S18. ^{1}H NMR spectrum of compound **TPE4S** (The normalization of the integrations appear to be for a single arm of the compound)**.**

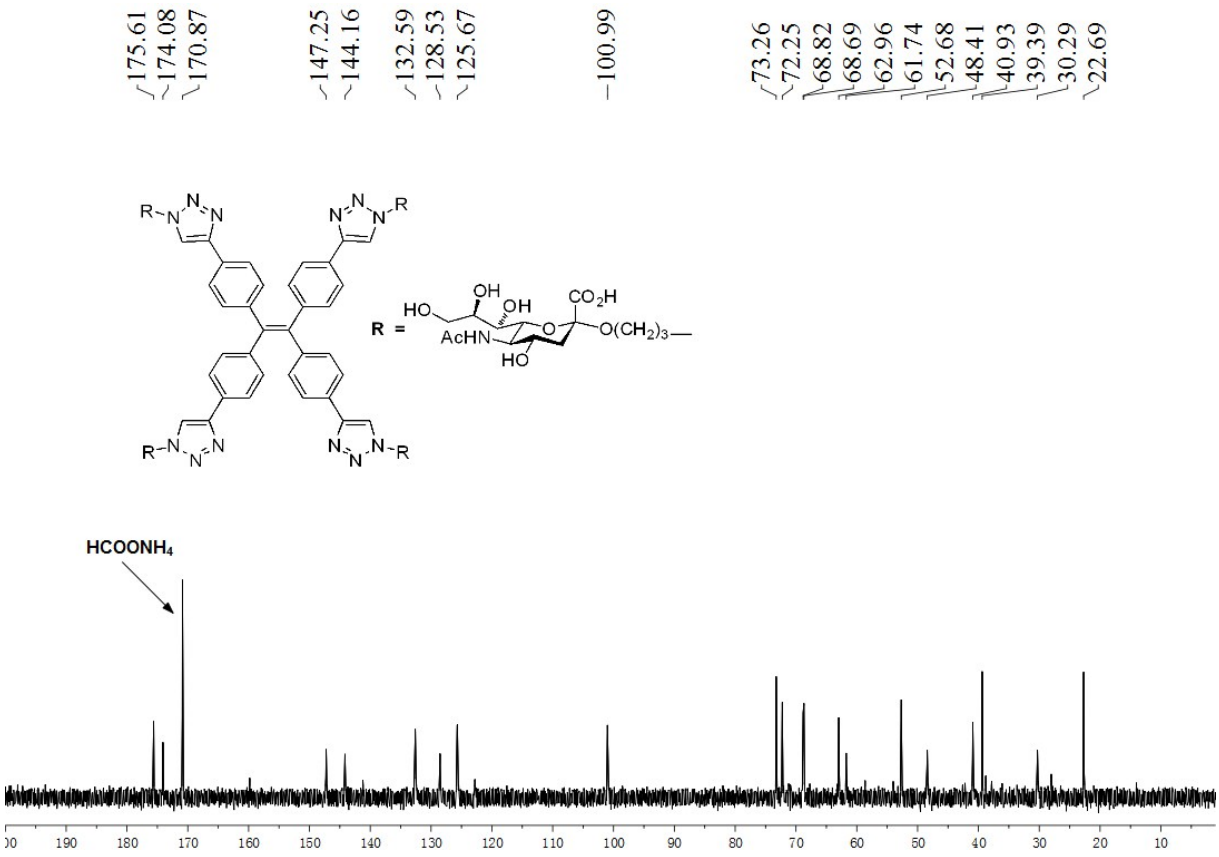


Figure S19. ^{13}C NMR spectrum of compound **TPE4S.**

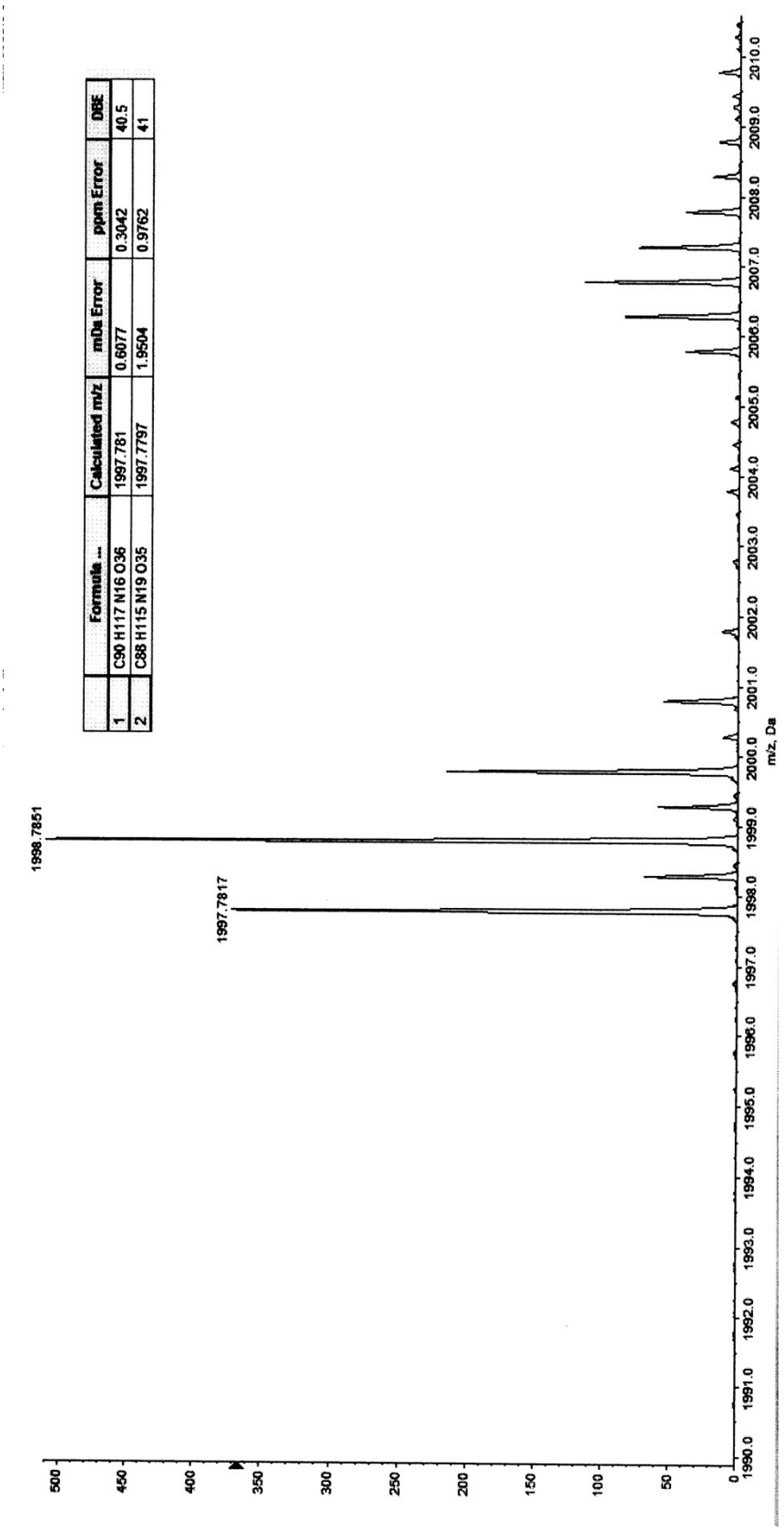


Figure S20. HRMS spectrum of compound **TPE4S**

8. References

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