Supporting Information

A Highly Selective Turn-on Fluorescent Probe Based on Semi-Cyanine for Detection of Nitroreductase and Hypoxic Tumor Cell Imaging

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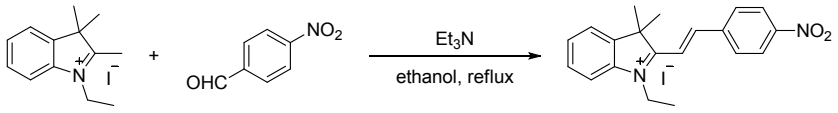
1. Materials and general methods

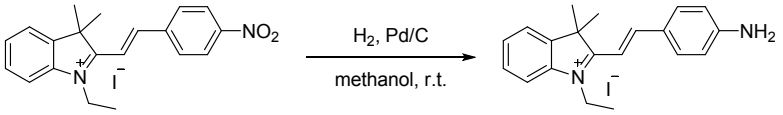
All chemical reagents and solvent were commercial products without further purification. Thin-layer chromatograph (TLC) was performed on silica gel plates. Column chromatography was performed using silica gel (Hailang, Qingdao) 200-300 mesh. ^{1}H and ^{13}C NMR spectra were recorded by employing a Bruker AV-400 spectrometer with chemical shifts expressed in parts per million (in DMSO-d_6, Me_4Si as internal standard). Electrospray ionization (ESI) mass spectrometry was performed in a HP 1100 LC-MS spectrometry.

2. Synthesis



semi-CyHP

To a mixture of *p*-nitrobenzaldehyde (0.151 g, 1.0 mmol) and 1,2,3-trimethyl-3-methyl-benzo indole iodized salt (0.315 g, 1.0 mmol) in ethanol (30.0 mL) was added 2~3 drops of triethylamine. Then the reaction solution were heated to reflux overnight. After cooling to room temperature, the solvent was removed under reduced pressure. Purification by flash column chromatography (silica gel, DCM:MeOH = 20:1). Yellow needles crystals (0.200 g, yield: 67%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.57-8.52 (m, 3H), 8.41 (d, J = 8.8 Hz, 2H), 8.05 (t, J = 4.4 Hz, 1H), 7.96 (t, J = 4.4 Hz, 1H), 7.90 (d, J = 16.4 Hz, 1H), 7.69 (t, J = 4.0 Hz, 2H), 5.76 (s, 1H), 4.83 (q, J = 7.2 Hz, 2H), 1.84 (s, 6H), 1.51 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 181.4, 150.0, 149.2, 144.4, 140.3, 140.2, 131.3, 130.1, 129.2, 124.0, 123.2, 116.3, 115.7, 54.9, 52.7, 25.1, 14.0; HRMS (ESI): calcd for $C_{20}H_{21}N_2O_2$ [M-I$^-$]$^+$ 321.1603, found 321.1595.



semi-CyHF

The mixture of **semi-CyHP** (0.448 g, 1.0 mmol) and catalytic amount of Pd/C (0.050 g) in methanol (50.0 mL) were under reaction at room temperature in hydrogen atmosphere (0.4 MPa) for 5 h. After reaction, the insoluble Pd/C and solvent were removed. Purification by flash column chromatography (silica gel, DCM:MeOH = 10:1). Red needles crystals (0.400 g, yield: 96%). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.28 (d, J = 14.4 Hz, 1H), 8.01 (d, J = 8.0 Hz, 2H), 7.77 (d, J = 7.2 Hz, 1H), 7.71 (d, J = 8.0 Hz, 1H), 7.54 (t, J = 8.0 Hz, 1H), 7.45 (d, J = 7.6 Hz, 1H), 7.24-7.18 (m, 3H), 6.74 (t, J = 8.4 Hz, 2H), 5.76 (s, 1H), 4.52 (q, J = 6.8 Hz, 2H), 1.73 (s, 6H), 1.37 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 178.7, 156.4, 155.0, 142.6, 140.7, 128.7, 127.3, 122.7, 122.3, 114.0, 113.2, 103.5, 54.8, 50.7, 26.4, 13.0; HRMS (ESI): calcd for $C_{20}H_{23}N_2$ [M-I$^-$]$^+$ 291.1861, found 291.1858.

3. Determination of quantum yield

The quantum yield of reduction product **semi-CyHF** was determined according to the literature.

$$\phi_1 = \frac{\phi_B I_1 A_B \lambda_{exB} \eta_1}{I_B A_1 \lambda_{ex1} \eta_B}$$

Where Φ is quantum yield; I is integrated area under the corrected emission spectra; A is absorbance at the excitation wavelength; λ_{ex} is the excitation wavelength; η is the refractive index of the solution; the subscripts 1 and B refer to the unknown and the standard, respectively. We

chose Fluorescein with 0.1 M NaOH solution as standard, which has the quantum yield of 0.95[1]. The quantum yields of **semi-CyHF** was calculated as 0.181.

4. Spectroscopic materials and methods

Double distilled water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 0.01 M PBS buffer (pH 7.0, 1% DMSO) at 37 ^{o}C. All pH measurements were made with a Sartorius basic pH-Meter PB-10. Absorption spectra were recorded using a Varian Cary 100 Bio UV-Visible spectrophotometer. Fluorescence spectra were recorded using a Varian Cary Eclipse scanning spectrofluorometer equipped with a Xenon flash lamp.

5. Nitroreductase activity assay

Nitroreductase activity experiments were employed recombinant forms of Nitroreductase (expressed in Escherichia coli. purchased from Sigma-Aldrich). Stock solutions of probe **semi-CyHP** and reduction product **semi-CyHF** were prepared in pure DMSO (1 mM) and diluted in phosphate buffered saline (pH 7.0) with 50 equiv. NADH. NBP was added to a final protein concentration of 17.5 μg/mL enzyme at 37 ºC, and emission intensity was collected from 500 nm to 750 nm with excitation at 490 nm.

Detection limit

The detection limit was calculated based on the fluorescence titration. The fluorescence enhancement of **semi-CyHP** was dose-dependent with respect to nitroreductase. The linear response ($y = 24.937 x + 66.704$ with $R^2 = 0.976$) of fluorescent intensity (y) with respect to the concentration (x) of nitroreductase was established. The lower detection limit (LDL) was calculated following equation. LDL = 3S/m (S is the ratio signal and noise, which is the standard deviation of blank measurements, n = 11; m is the slope of linear equation). The detection limit was determined to be 40 ng/mL.

6. Cell viability assay

Cell proliferation was evaluated by MTT assay. A549 cells were seeded in 96-well plates at a density of 1×104 cells per well and incubated overnight, and then treated with probe at different concentration (1.0μM, 2.0μM, 5.0μM, 10.0μM, 15.0μM, 20.0μM) for 48 h. After that, 20 μL MTT solution (5 mg/mL) was added to each well and incubated for another 4 h. 100 μL DMSO were added to dissolve the crystals in each well. Absorbance values were measured at 490 nm with PE Enspire multifunction microplate reader (USA). The cell viability was calculated according to the following equation: Cell viability (%) = A490 (sample)/A490 (control) ×100.

7. Hypoxia cell assay

Lung carcinoma cell line A549 cells were grown in F12 supplemented with 10% FBS and 1% L-Glutamine. Cells were incubated in a 5% CO_2 humidified incubator at 37 ^{o}C and typically passaged with sub-cultivation ratio of 1:4 every two days. A549 cells were seeded in 24-well plates in F-12 culture medium. For fluorescence microscopy, the cells were incubated under normoxic (75% N_2, 5% CO_2, 20% O_2) and hypoxic (94% N_2, 5% CO_2, 1% O_2) condition for 21 h at 37 ^{o}C. Then the cells were washed twice with D-hanks buffer (pH 7.0)and treated with 5μM probe in FBS-free F-12 culture medium for 1 h under hypoxia condition. Before taken images, A549 cells were washed three times with D-hanks buffer (pH 7.0). Fluorescence imaging was performed with Nikon Tis fluorescence microscope. The fluorescent field was collected with 1 s exposure time and bright field with 600 ms.

8. Data

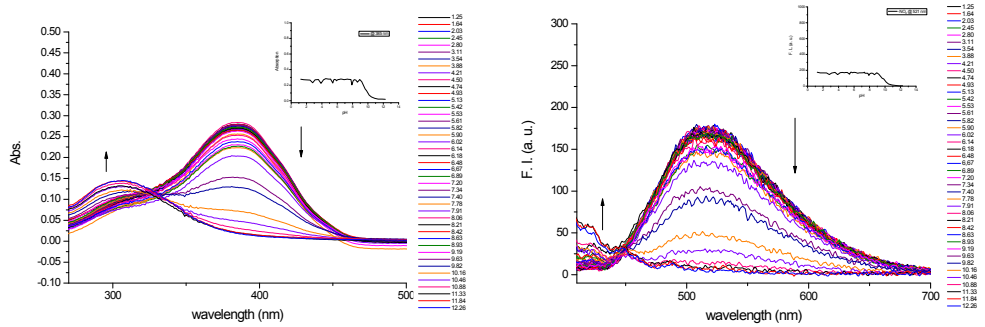


Fig. S1. The pH titration of fluorophore **semi-CyHP** (10.0 μM) in H_2O (1% DMSO as co-solution).

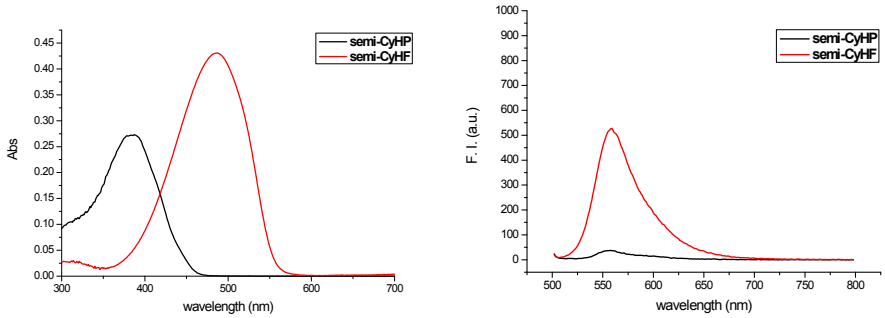


Fig. S2. The UV absorption spectra and fluorescent emission spectra probe. **semi-CyHP** (10.0 μM, black line), and **semi-CyHF** (10.0 μM , read line) in 0.01 M PBS buffer (pH 7.0, 1% DMSO). Excitation wavelength was 490 nm; slit: 10, 10 nm.

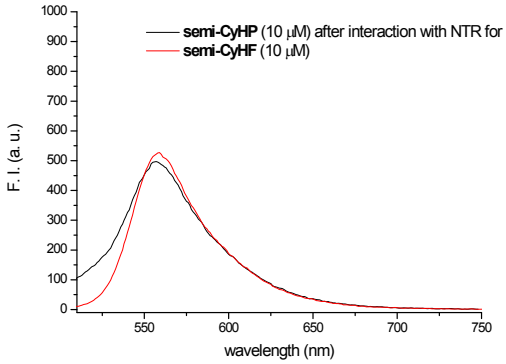


Fig. S3. The conversion rate of **semi-CyHP** (10 μM) after interaction with NTR for 70 min. The measurements were performed in 0.01 M PBS buffer (pH 7.0, 1% DMSO) with NTR (17.5μg/mL) and 50 equivalent NADH at 37 °C over an incubation time of 70 min. The fluorescent intensity data were collected after reaction with excitation at 490 nm. Silt: 10, 10 nm. It was compared with the reduction product **semi-CyHF** (10 μM) as indicated in the figure for the calculation of the yield.

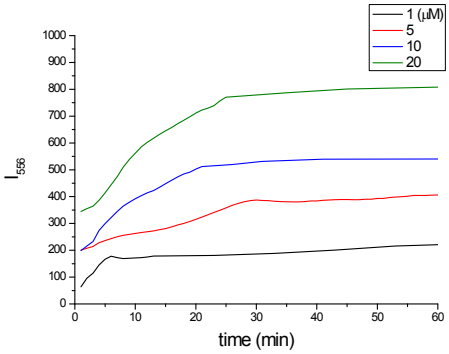


Fig. S4. Plots of I_{556} with varied concentrations of **semi-CyHP**: 1.0, 5.0, 10.0 and 20.0 μM. The measurements were performed in 0.01 M PBS buffer (pH 7.0, 1% DMSO) with NTR (17.5μg/mL) and 50 equivalent NADH at 37 ºC over an incubation time of 60 min. The fluorescent intensity data were collected after certain time intervals as indicated in the figure with excitation at 490 nm. Silt: 10, 10 nm.

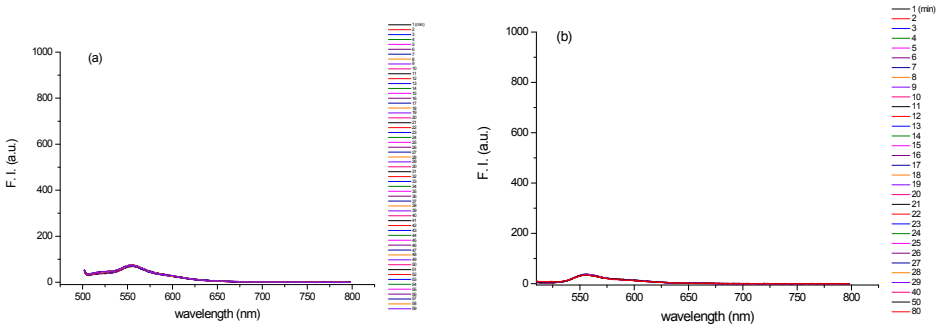


Fig. S5. The Reaction–time profile of probe **semi-CyHP** (10 μM) in the presence of NTR (17.5 μg/mL) or 50 equiv. of NADH in 0.01 M PBS buffer (pH 7.0, 1% DMSO). Excitation wavelength was 490 nm.

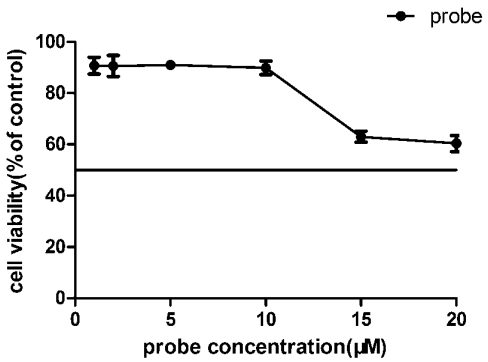
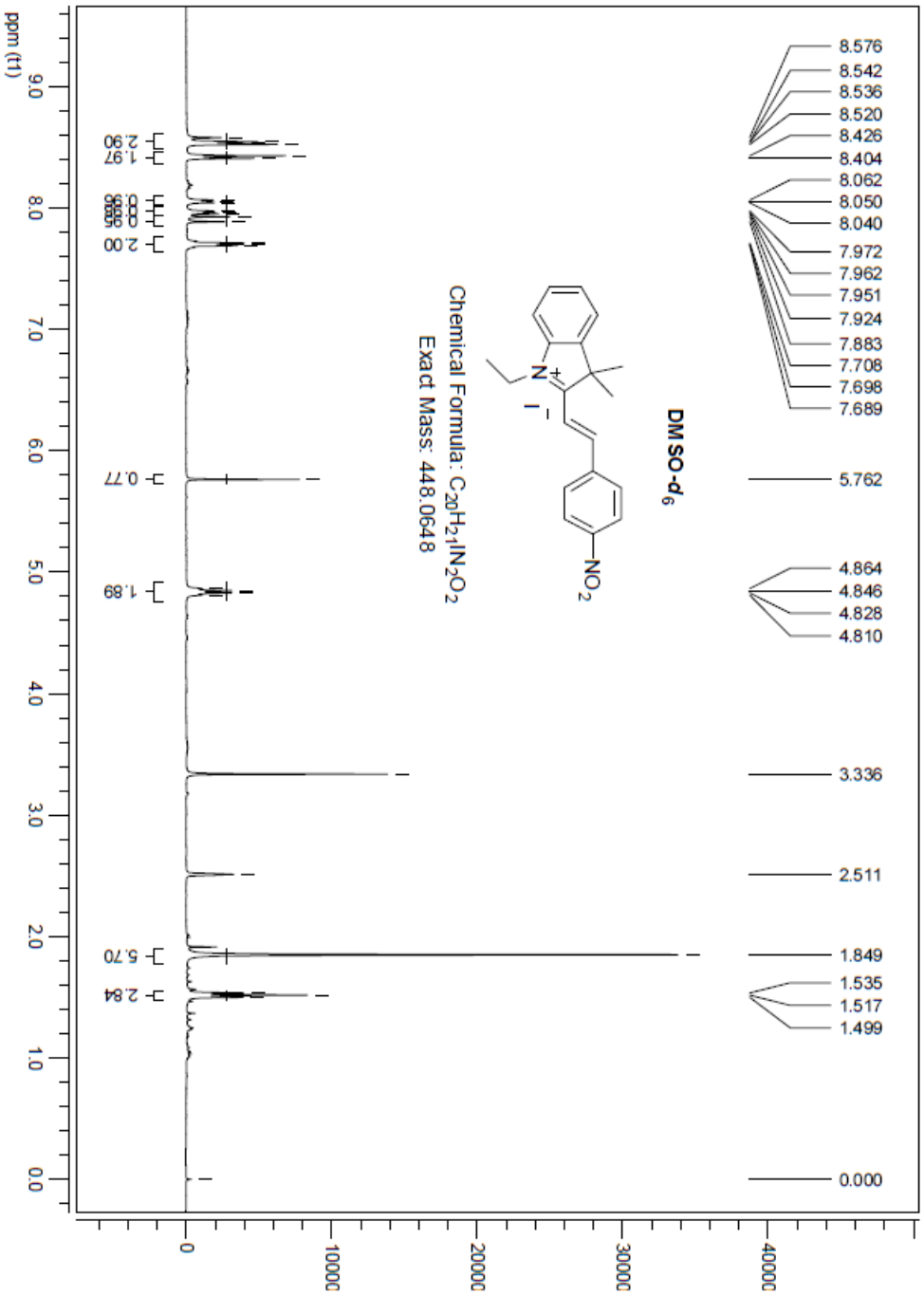


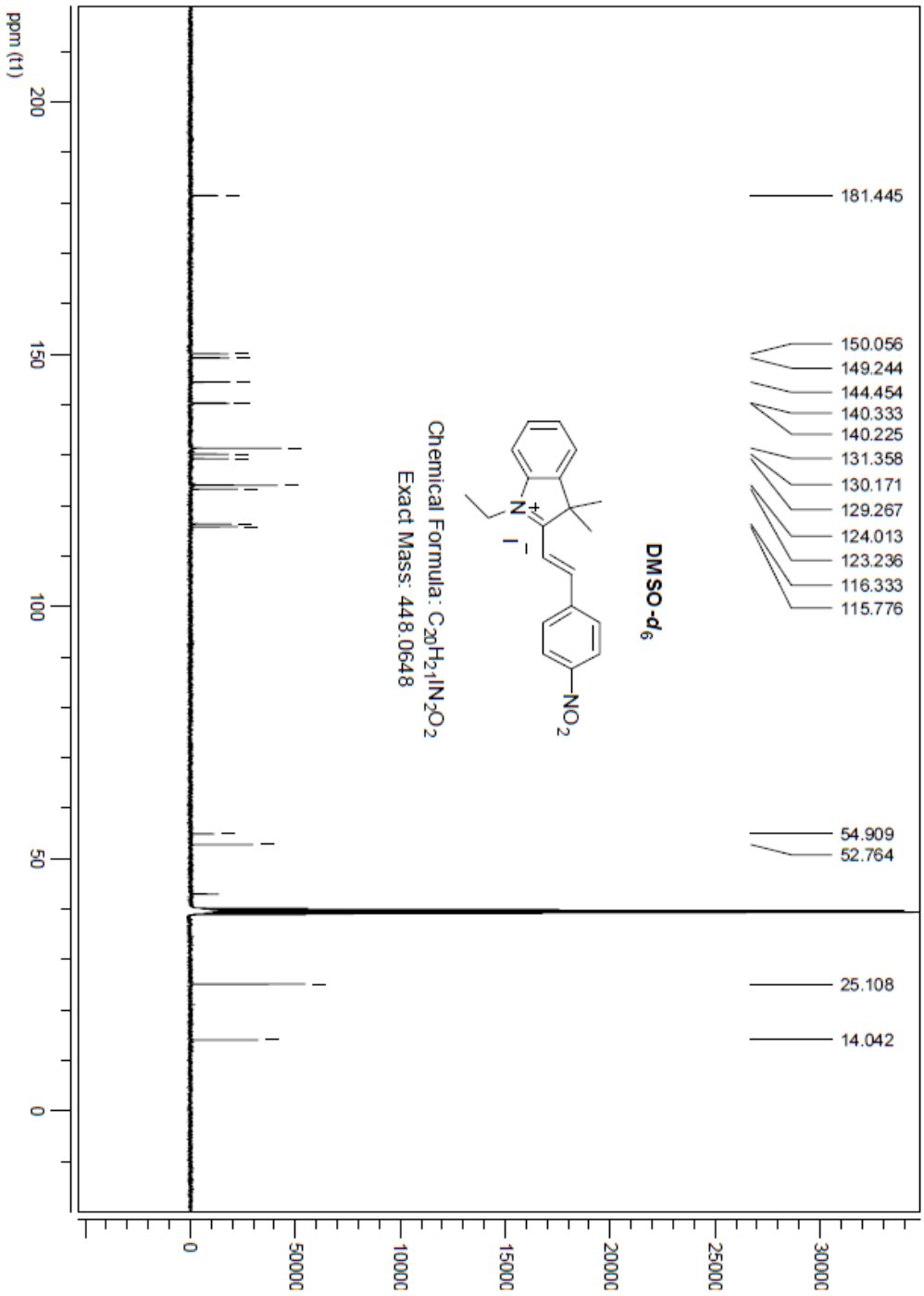
Fig. S6. Cell Viability Assays of probe **semi-CyHP**.

9. NMR and MS spectra

^{1}H NMR for **semi-CyHP**



^{13}C NMR for **semi-CyHP**



Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 30.0 mDa / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
90 formula(e) evaluated with 7 results within limits (up to 1 closest results for each mass)
Elements Used:
C: 0-59 H: 0-81 N: 0-4 O: 0-3

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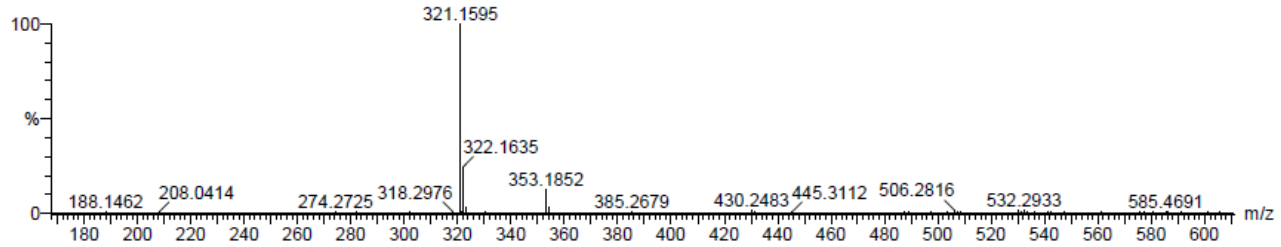
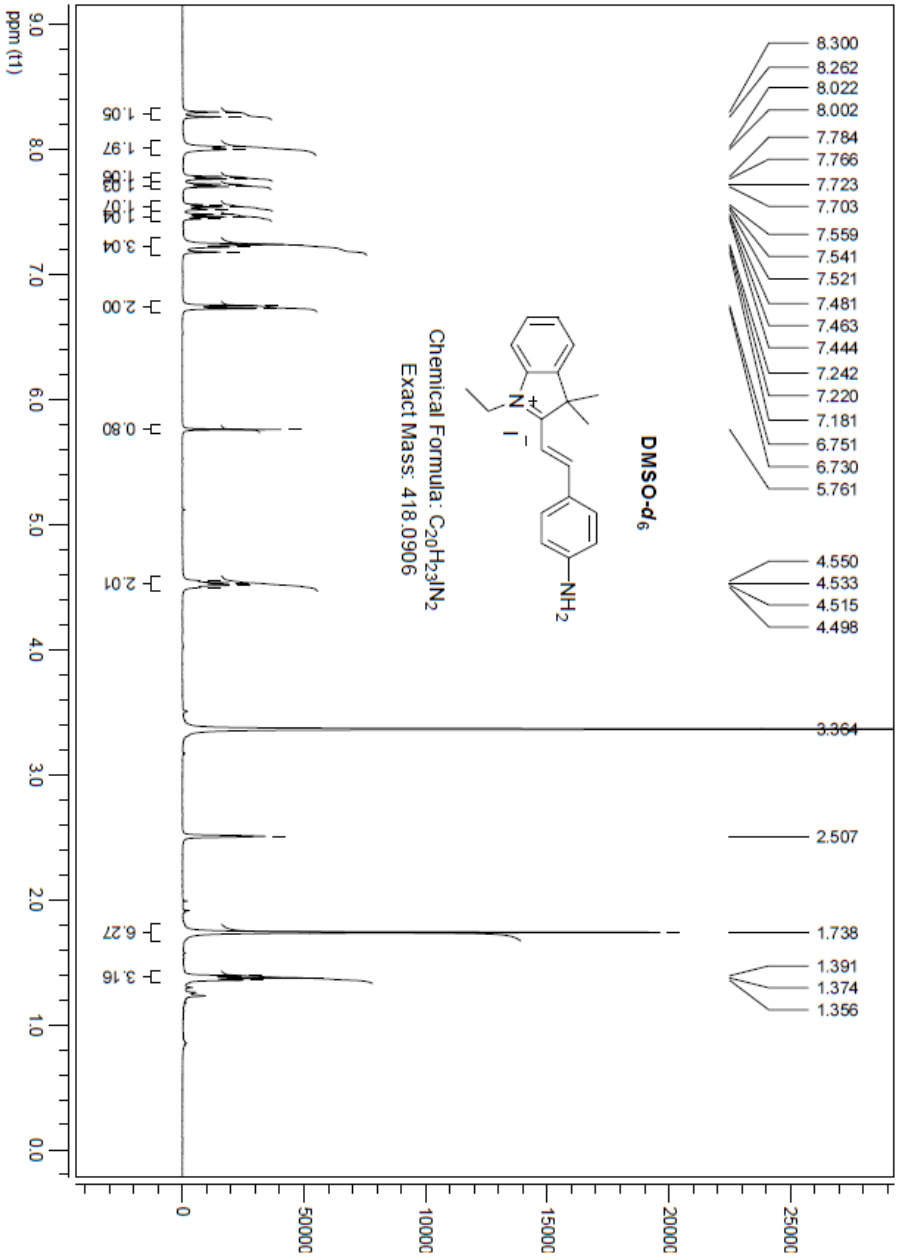
ZWP-XYS-29 65 (0.502) Cm (62:69) 1: TOF MS ES+ 2.69e+004



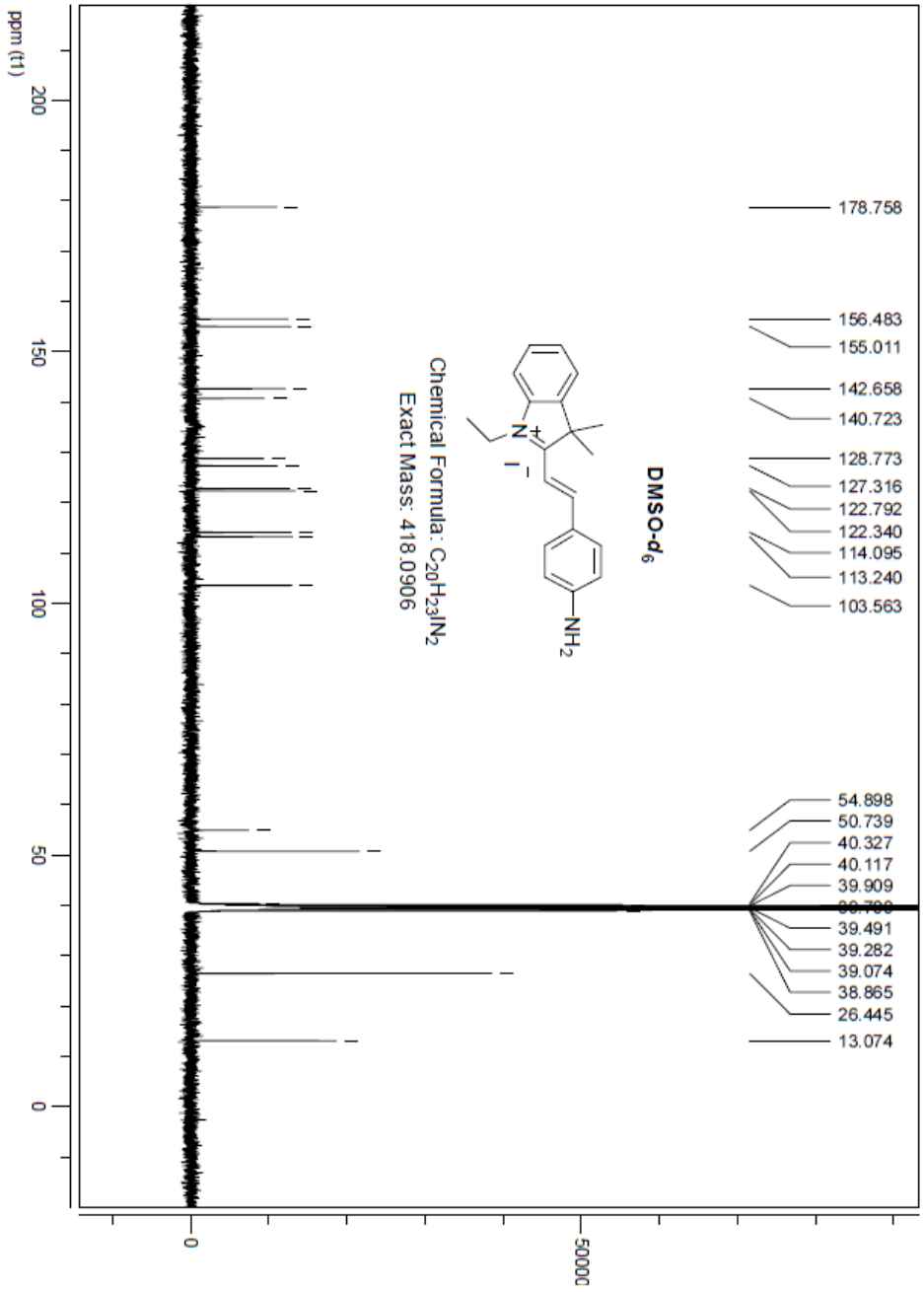
Minimum:				-1.5			
Maximum:		30.0	50.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
321.1595	321.1603	-0.8	-2.5	11.5	255.7	0.0	C20 H21 N2 O2

HRMS for **semi-CyHP**

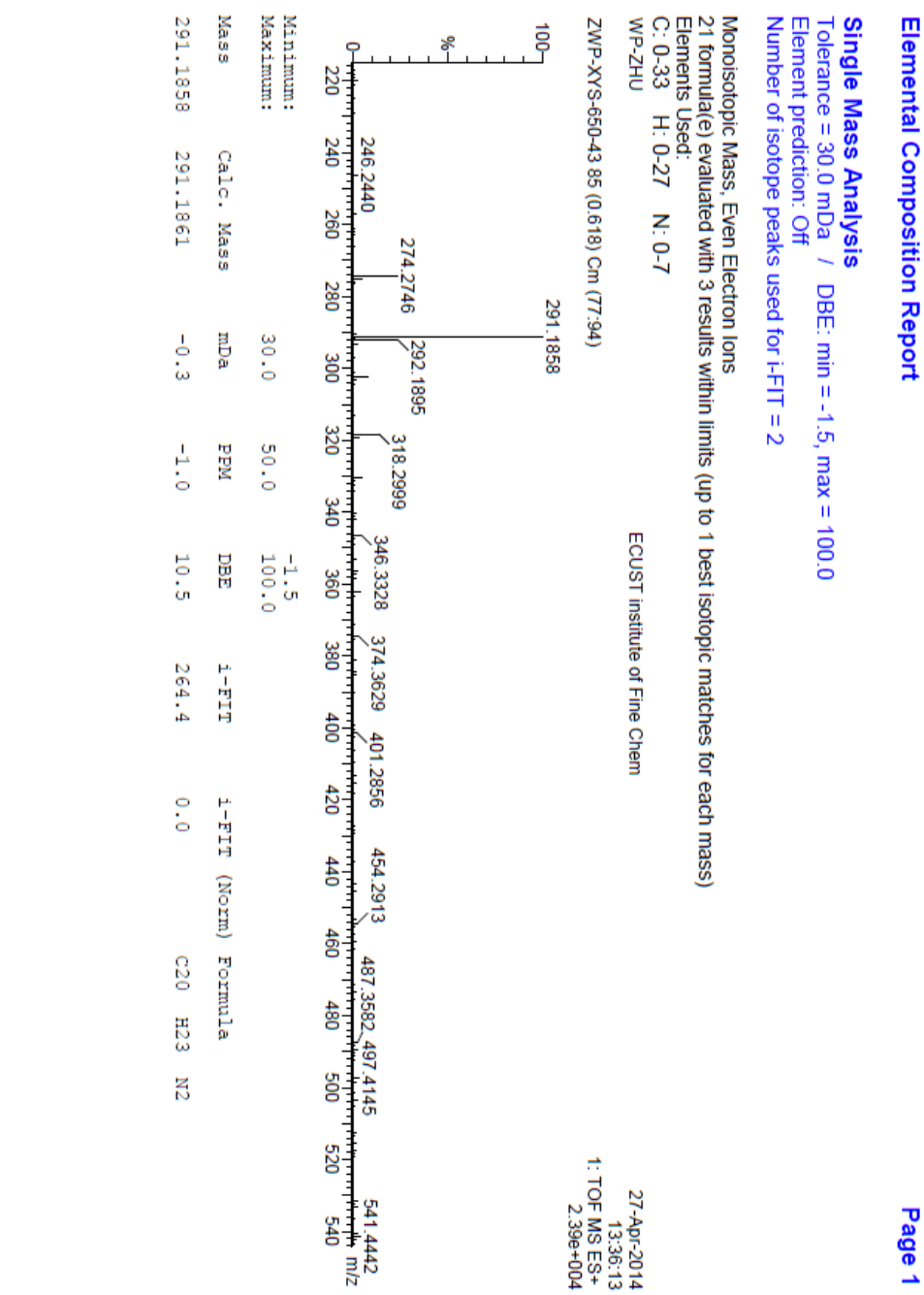
^{1}H NMR for **semi-CyHF**



^{13}C NMR for **semi-CyHF**



HRMS for **semi-CyHF**



10. Reference

1. Lakowicz, J.R. *Principles of Fluorescence Spectroscopy,* Springer Maryland, 3rd edn., 2006, pp. 9-12.