A PNIPAM-Based Fluorescent Nanothermometer with Ratiometric Readout

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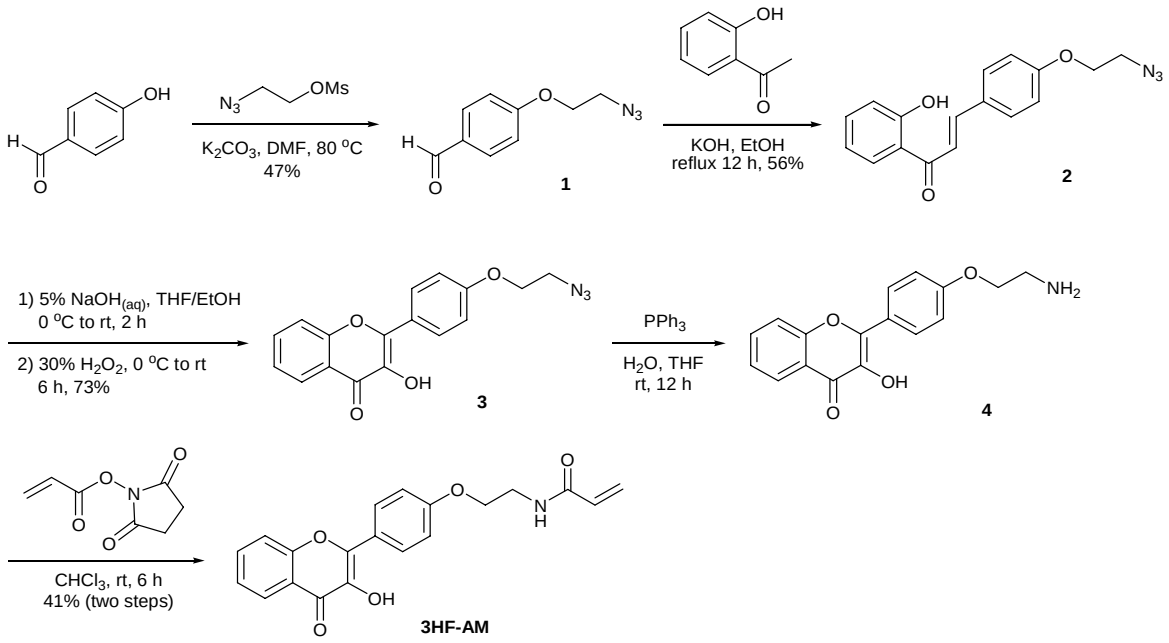
I. Materials and apparatus

N, *N*′-Methylenebisacrylamide (MBAM), ammonium persulfate (APS), sodium dodecyl sulfate (SDS), *N*, *N*, *N*′, *N*′-tetramethylethylenediamine (TMEDA) and 2, 2'-azobis(2-methylpropionitrile) (AIBN) were obtained from Acros chemicals. 2, 2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) and *N*-isopropylacrylamide (NIPAM) were purchased from Aldrich and TCI chemicals, respectively. NIPAM was purified by recrystallization from *n*-hexane prior to use. All other reagents and solvents were used without further purification.

Proton nuclear magnetic resonance (^{1}H NMR) spectra and carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on Varian Mercury Plus 400 and Bruker AVANCE 400 spectrometers. Melting points were measured using a Fargo MP-1D melting point apparatus without correction. Infrared spectra were recorded on Varian 640-IR spectrometer. Mass spectra with an electrospray ionization (ESI) system were determined on a WATERS LCT Premier Xe spectrometer. The centrifugation was operated on eppendof 5810R. UV-visble absorption spectra were recorded at 25 °C using a Hewlett-Packard 8453A diode array spectrometer under the control of a Pentium PC running the manufacturer-supplied software package. Turbidity was obtained from Perkin Elmer Lambda 950 UV/Vis spectrometer with PTP-1+1 Peltier temperature programmer element. Fluorescence spectra were recorded on a Hitachi F-4500 spectrofluorometer with Specac variable temperature cell 6100+ equipped with temperature controller and NaCl window for the jacket. The TEM specimens were prepared by settling a drop (10 uL) of polymer aqueous solution (0.01 w/v%) on formvar-coated carbon grids for 5 minutes, removing the excess solution by filter papers followed by the negative staining with a drop (10 uL) of 1% uranyl acetate (UA) aqueous solution for 30 seconds, removing the excess UA solution, and being air-dried. The TEM images were obtained on Hitachi 7650 transmission electron microscope at a voltage of 77 kV. The hydrodynamic diameters were obtained by dynamic light scattering measurement with Zetasizer Nano Particle Analyzer ZS.

II. Experimental procedures

Scheme S1. Synthetic scheme of 3HF-AM



Synthetic procedures and physical data

4-(2-azidoethoxy)benzaldehyde (1)[1]

4-Hydroxybenzaldehyde (5.610 g, 45.94 mmol) and potassium carbonate (9.520 g, 68.88 mmol) were added to a solution of 2-azidoethyl methanesulfonate (57.42 mmol), which was in-situ prepared by reacting 2-azidoethanol with methanesulfonyl chloride, in *N*, *N*-dimethylformamide (60 mL) at room temperature and the reaction mixture was stirred for 12 h at 80 °C. The resulting mixture was poured into water (300 mL) and extracted with ether (4×30 mL). The combined organic layers were washed with brine (20 mL), dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel with ethyl acetate-hexane (1:3) as an eluent to yield 4.103 g (21.45 mmol) of **1** as a light yellow oil in 47%: TLC (ethyl acetate/hexane (1:3)) R_f = 0.21; IR (KBr) 2736 (ν C(=O)H) cm^{-1}, 2111 (ν N_3) cm^{-1}, 1694 (ν C=O) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.83 (s, 1H), 7.79 (d, J = 8.8 Hz, 2H), 6.97 (d, J = 8.8 Hz, 2H), 4.18 (t, J = 4.8 Hz, 2H), 3.60 (t, J = 4.8 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 190.3, 162.8, 131.7, 130.1, 114.6, 67.1, 49.9; ESI-HRMS: calculated for $C_9H_{10}N_3O_2$ ($[M+H]^+$) 192.0768; found 192.0768.

(2*E*)-3-[4-(2-azidoethoxy)phenyl]-1-(2-hydroxyphenyl)prop-2-en-1-one (2)

To a solution of **1** (2.734 g, 14.30 mmol) and 2-hydroxyacetophenone (1.9 mL, 15.78 mmol) in ethanol (10 mL) was added potassium hydroxide (2.810 g, 50.08 mmol) in ethanol (17.3 mL), and the reaction mixture was stirred for 12 h at 78 °C. The resulting mixture was poured into saturated ammonium chloride solution (100 mL) and extracted with dichloromethane (3×50 mL). The combined organic layers were washed with brine (15 mL), dried over $MgSO_4$, filtered, and

concentrated under reduced pressure. The residue was purified by recrystallization from ethyl acetate to afford 2.478 g (8.01 mmol) of **2** as a yellow solid in 56%: mp 120~121 ^{o}C; TLC (ethyl acetate/hexane (1:3)) R_f = 0.42; IR (KBr) 2110 (ν N_3) cm^{-1}, 1634 (ν C=O) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.87 (s, 1H), 7.91-7.89 (m, 1H), 7.88 (d, J = 15.2 Hz, 1H), 7.62 (d, J = 8.8 Hz, 2H), 7.53 (d, J = 15.2 Hz, 1H), 7.50-7.45 (m, 1H), 7.02-7.00 (m, 1H), 6.96 (d, J = 8.8 Hz, 2H), 6.93-6.90 (m, 1H), 4.20 (t, J = 4.8 Hz, 2H), 3.63 (t, J = 4.8 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 193.3, 163.3, 160.3, 144.9, 136.1, 130.4, 129.4, 127.9, 120.0, 118.7, 118.5, 117.9, 115.0, 67.1, 50.1; ESI-HRMS: calculated for $C_{17}H_{14}N_3O_3$ ($[M-H]^-$) 308.1041; found 309.1035.

2-[4-(2-azidoethoxy)phenyl]-3-hydroxy-4H-chromen-4-one (3)

To a solution of **2** (375 mg, 1.21 mmol) in the co-solvents of THF (4 mL) and ethanol (12 mL) was added 5% sodium hydroxide aqueous solution (4 mL) at 0 ^{o}C, and then warmed up to room temperature in 2 h. To the reaction mixture was added 30% hydrogen peroxide (420 μL) at 0 ^{o}C, and then warmed up to room temperature followed by stirring at room temperature for 6 h. The resulting mixture was poured into 1N HCl solution (20 mL) and extracted with dichloromethane (3×10 mL). The combined organic layers were washed with brine (10 mL), dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by recrystallization from ethyl acetate to afford 286 mg (0.89 mmol) of **3** as a light yellow solid in 73%: mp 125~126 ^{o}C; TLC (ethyl acetate/hexane (1:3)) R_f = 0.18; IR (KBr) 2110 (ν N_3) cm^{-1}, 1602 (ν C=O) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.23-8.21 (m, 3H), 7.67 (ddd, J = 8.0, 6.8, 1.6 Hz, 1H), 7.56-7.54 (m, 1H), 7.39 (ddd, J = 8.0, 6.8, 1.2 Hz, 1H), 7.04 (d, J = 9.2 Hz, 2H), 6.99 (bs, 1H), 4.22 (t, J = 5.2 Hz, 2H), 3.64 (t, J = 5.2 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.9, 159.4, 155.1, 144.8, 137.6, 133.3, 129.4, 125.3, 124.3, 124.1, 120.6, 118.0, 114.5, 67.0, 50.1; ESI-HRMS: calculated for $C_{17}H_{14}N_3O_4$ ($[M+H]^+$) 324.0979; found 324.0979.

2-[4-(3-hydroxy-4-oxo-4H-chromen-2-yl)phenoxy]ethylacrylamide (3HF-AM)

Water (90 μL) was added to a solution of **3** (202 mg, 0.62 mmol) and triphenylphosphine (196 mg, 0.75 mmol) in THF (4 mL), and the reaction mixture was stirred at room temperature for 12 h. The resulting mixture was concentrated under reduced pressure, and the yellowish residue was purified by triturated with a large amount of dichloromethane (200 mL) to give crude **4** as a yellow solid, which was taken up in chloroform (20 mL). *N*-acryloxysuccinimide (109 mg, 0.64 mmol) was added to the aforementioned suspension of crude **4** at room temperature, and the reaction mixture was stirred at room temperature for 6 h. The resulting mixture was poured into water (40 mL) and extracted with dichloromethane (3×20 mL). The combined organic layers were washed with brine (10 mL), dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with dichloromethane-methanol (19:1) as an eluent to yield 89 mg (0.25 mmol) of **3HF-AM** as a pale yellow solid in 41%: mp >197 ^{o}C (decompose); TLC (dichloromethane-methanol (19:1)) R_f = 0.44; IR (KBr) 3300 (ν NH) cm^{-1}, 1601 (ν C=O) cm^{-1}, 1562 (ν C=O) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD) δ 8.19-8.14 (m, 3H),

7.66-7.62 (m, 1H), 7.53 (d, J = 8.4 Hz, 1H), 7.38-7.34 (m, 1H), 6.99 (d, J = 9.2 Hz, 1H), 6.74 (bs, 1H), 6.25 (dd, J = 16.8, 1.2 Hz, 1H), 6.11 (dd, J = 16.8, 10.0 Hz, 1H), 5.63 (dd, J = 10, 1.2 Hz, 1H), 4.12 (t, J = 4.8 Hz, 2H), 3.74-3.70 (m, 2H); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 172.7, 165.0, 159.6, 154.5, 145.5, 138.3, 133.6, 131.6, 129.5, 125.5, 124.8, 124.5, 123.8, 121.4, 118.4, 114.6, 66.5, 38.3; ESI-HRMS: calculated for $C_{25}H_{26}NO_5S_2$ ([M-H]$^-$) 350.1034; found 350.1032.

Fluorescence behaviors of 3HF-AM monomer (1×10^{-5} M) in various proportions of DMSO and water and the corresponding fluorescent images

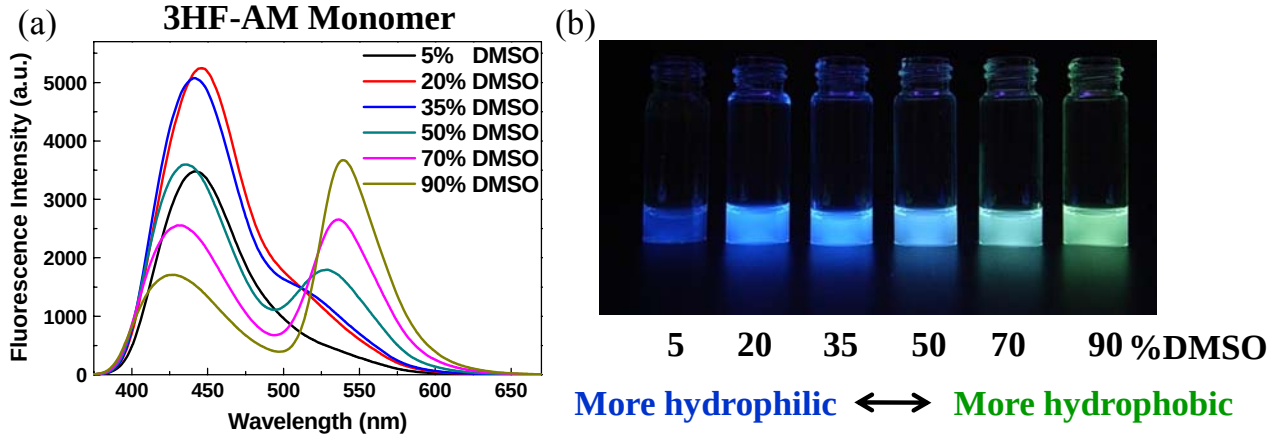


Fig. S1 (a) Overlaid fluorescence spectra of **3HF-AM** monomer (1×10^{-5} M) in various proportions of DMSO and water mimicking different hydrophilicity. (b) Corresponding fluorescent photo images.

Procedure for the preparation of Thermo-3HF

Thermo-3HF was prepared using a modified emulsion polymerization method delineated by Uchiyama *et al.*[2] NIPAM (226 mg, 2 mmol), MBAM (3 mg, 0.02 mmol), and SDS (73 mg, 0.25 mmol) were weighed to a three-neck round bottle equipped with a thermocouple and a condenser, and removed the air in the system via high vacuo and then refilled with the inert gas argon. A solution of **3HF-AM** (7 mg, 0.02 mmol) in degassed DMF and degassed deionized water was added to the Ar-filled three-neck round bottle to dissolve NIPAM, MBAM, and SDS. To the resulting mixture was added TMEDA (9 μL, 0.06 mmol) and warmed up to 70 ^{o}C for 5 minutes. A solution of ammonium persulfate (128 mg, 0.56 mmol) in degassed water (2 mL) was added to initiate the emulsion polymerization and the reaction was carried out at 70 ^{o}C for 4 h under argon atmosphere with a stirring rate of 260 rpm. The resulting mixture was poured into deionized water (400 mL) and the polymer was precipitated by salting-out. The precipitates were collected by filtration and further washed with clean saturated brine. Excess brine was subsequently removed by centrifugation to afford a light yellow flake, which was further purified by the size exclusion chromatography on G-100 gel with deionized water as an eluent. The desired fractions were lyophilized to afford copolymers as pale yellow cotton-like solids.

Table S1. Optimization of cosolvent compositions for the polymerization reactions to obtain the nanogels with thermo-sensing range around the body temperature.

Batch[a]	Cosolvent composition		Polymerization Yield	3**HF-AM** content[b]	Thermo-sensing range (°C)
	H_2O (mL)	DMF (mL)			
1	20	0	52%	0.10%	32-37
2	15	5	41%	0.18%	32-39
3 **(Thermo-3HF)**	13	7	45%	0.55%	33-41
4	10	10	44%	0.23%	39-47
5	8	12	40%	0.17%	40-48

[a] NIPAM (226 mg, 2 mmol), MBAM (3 mg, 0.02 mmol), **3HF-AM** (7 mg, 0.02 mmol), APS (128 mg, 0.56 mmol), TMEDA (9 μL, 0.06 mmol), SDS (73 mg, 0.25 mmol); reaction temperature: 70 °C; reaction time: 4 h; stirring rate: 260 rpm

[b] The content of **3HF-AM** in batch **1-5** polymers was derived from the absorbance of nanogel in DMSO-*d6* as compared to **3HF-AM** monomer in DMSO-*d6* (ε= 12016 $M^{-1}cm^{-1}$ at 356 nm). The content of **3HF-AM** in **Thermo-3HF** was estimated to be 0.55%. The lower content of **3HF-AM** in **Thermo-3HF** was attributed to lower solubility of **3HF-AM** in the reaction medium.

Physical characterizations of Thermo-3HF

The ^{1}H NMR spectrum of the synthesized **Thermo-3HF** in CD_3OD was shown at Fig. S2, showing a surfactant-free polymer nature.

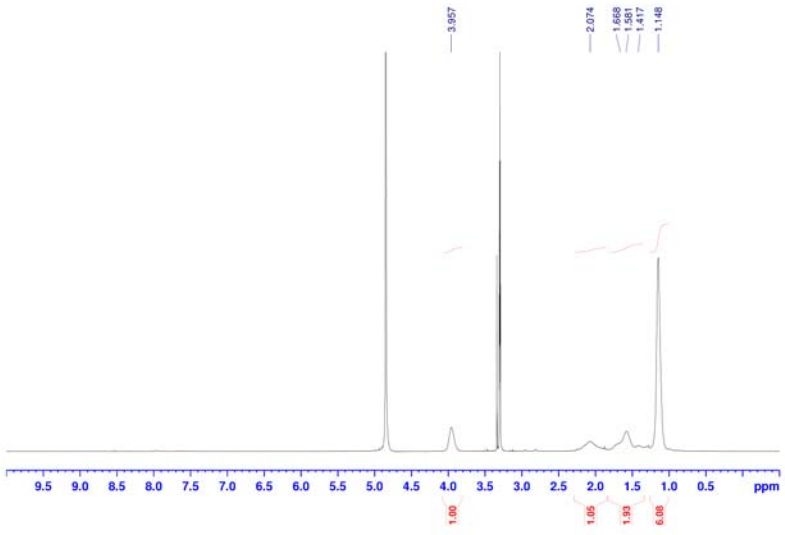


Fig. S2 ^{1}H NMR spectrum (400 MHz) of **Thermo-3HF** in CD_3OD

Hydrodynamic diameter (z-average diameter)

The variable temperature size experiments were carried out using Zetasizer Nano Particle Analyzer ZS in Temperature-Trend mode. The size analysis was conducted in an aqueous 0.002 w/v% nanogel solution. At each temperature, three measurements were performed and they are within the standard deviation of 10%.

Variable temperature turbidity measurements

The turbidity measurements were conducted in an aqueous 0.01 w/v% nanogel solution. The turbidity was estimated from the absorbance at 500 nm of the absorption spectra, which avoids the interference of **3HF-AM** absorption.

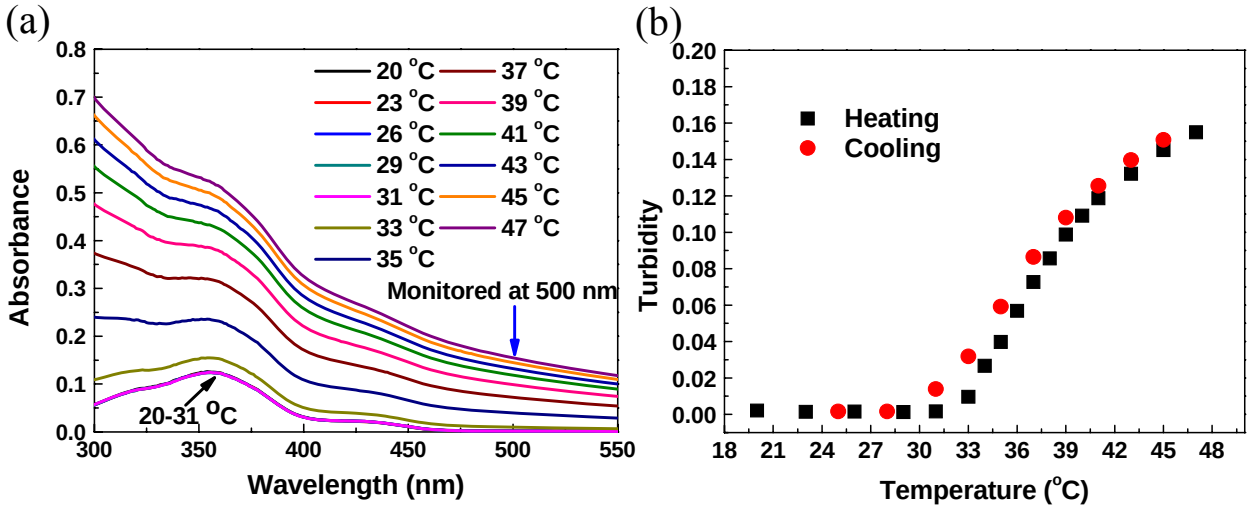


Fig. S3 (a) Overlaid absorption spectra of **Thermo-3HF** in aqueous solution (0.01 w/v%) at various temperatures. (b) Correlation between the temperatures and the turbidities of **Thermo-3HF**, monitored at 500 nm. ■: heating process; ◆: cooling process.

III. Spectroscopic studies

Unless specifically mentioned, all the spectroscopic studies were conducted in an aqueous 0.01 w/v% nanogel solution. All the spectroscopic data were acquired after the temperature equilibrium was reached and maintained at the equilibrium for at least 5 minutes. In addition, the sample holder was surrounded by a water-cooled system in the cooling experiments.

Variable temperature fluorescence measurements and data processing to associate with the ratiometric representation

The nanogel solutions were excited at 355nm and the emissions were collected from 376 to 670 nm,

with 5nm slits for excitation and emission, respectively. The data processing follows the following steps: (1) Convert the original wavelength-based fluorescence spectra into wavenumber-based fluorescence spectra. (2) Integrate the intensities from 26.6 Kcm^{-1} to the middle wavevalley as ESICT band energy and from the middle wavevalley to 14.9 Kcm^{-1} as ESIPT band energy. (3) The ratios of integrated intensities are calculated by taking the ESIPT band energy divided by ESICT band energy. (4) Normalize the ratios by scaling the maximum values to one. (For example, in the case of **Thermo-3HF,** scale the ratio of integrated intensities at 52 ^{o}C to one).

Variable temperature fluorescence measurements under physiological condition

The variable temperature fluorescence measurements were conducted in pH = 7.4 PBS buffer solution.

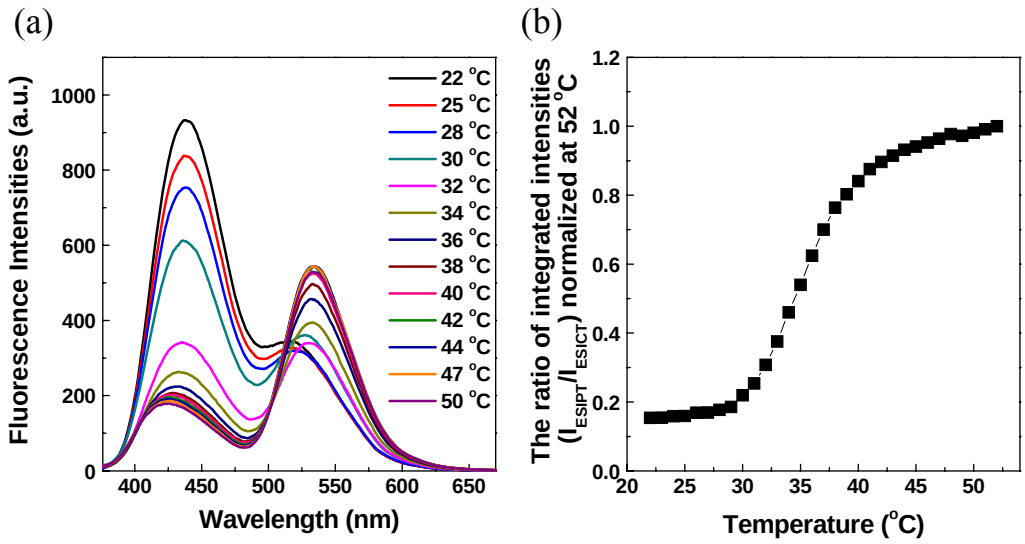


Fig. S4 (a) Overlaid fluorescence spectra of **Thermo-3HF** in pH = 7.4 PBS buffer solution (0.01 w/v%) at various temperatures; Excitation wavelength: 355 nm. Every spectrum was acquired after reaching equilibrium. (b) A correlation between the temperatures and normalized ratios of integrated intensities of ESIPT and ESICT bands of **Thermo-3HF**. The data used in (b) is derived from the spectra of (a).

References

1. X. Zhang, Y. Xiao and X. Qian, *Org. Lett.* 2008, **10**, 29.
2. C. Gota, K. Okabe, T. Funatsu, Y. Harada and S. Uchiyama, *J. Am. Chem. Soc.*, 2009, **131**, 2766.

IV. ^{1}H and ^{13}C spectra of monomer precursors and 3HF-AM

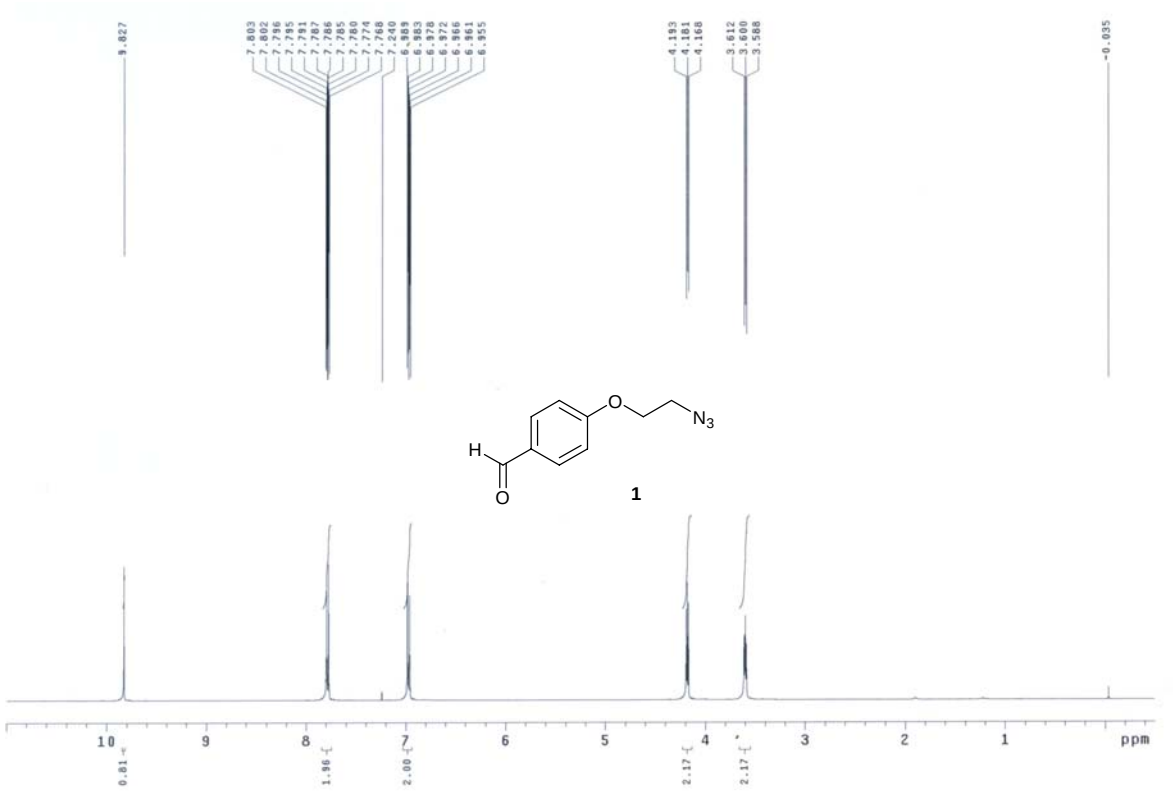


Fig. S5 ^{1}H NMR spectrum (400 MHz) of compound **1** in $CDCl_3$

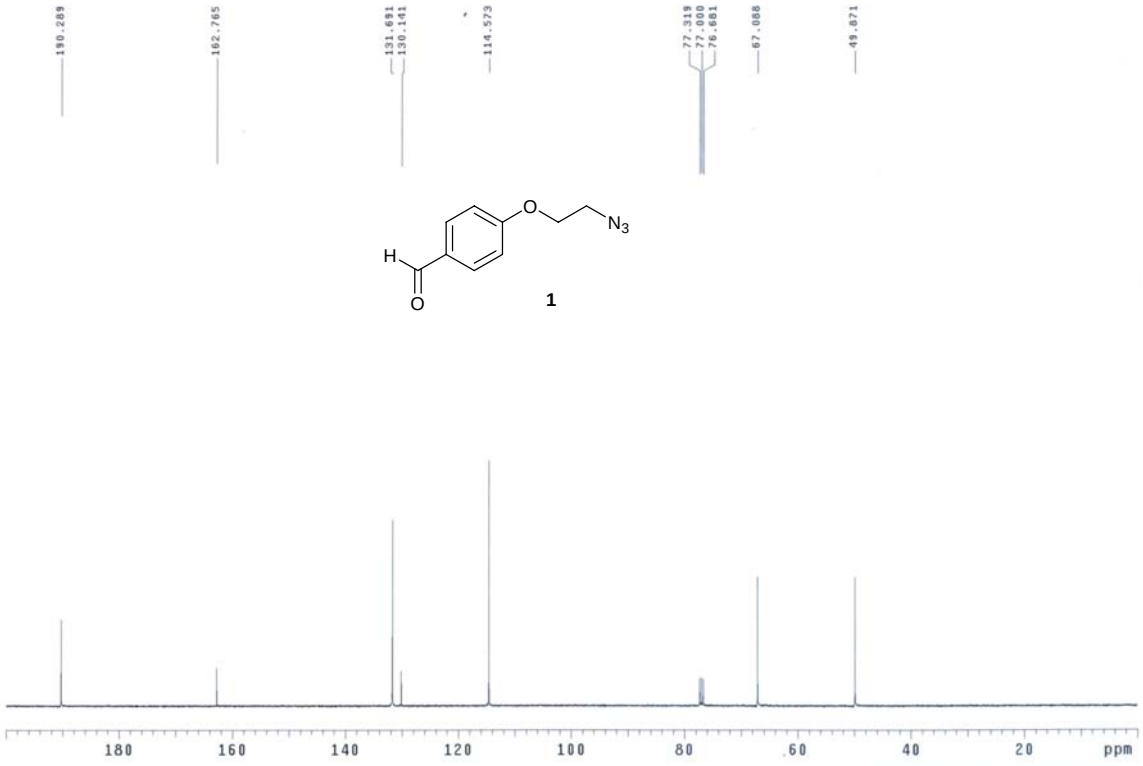


Fig. S6 ^{13}C NMR spectrum (100 MHz) of compound **1** in $CDCl_3$

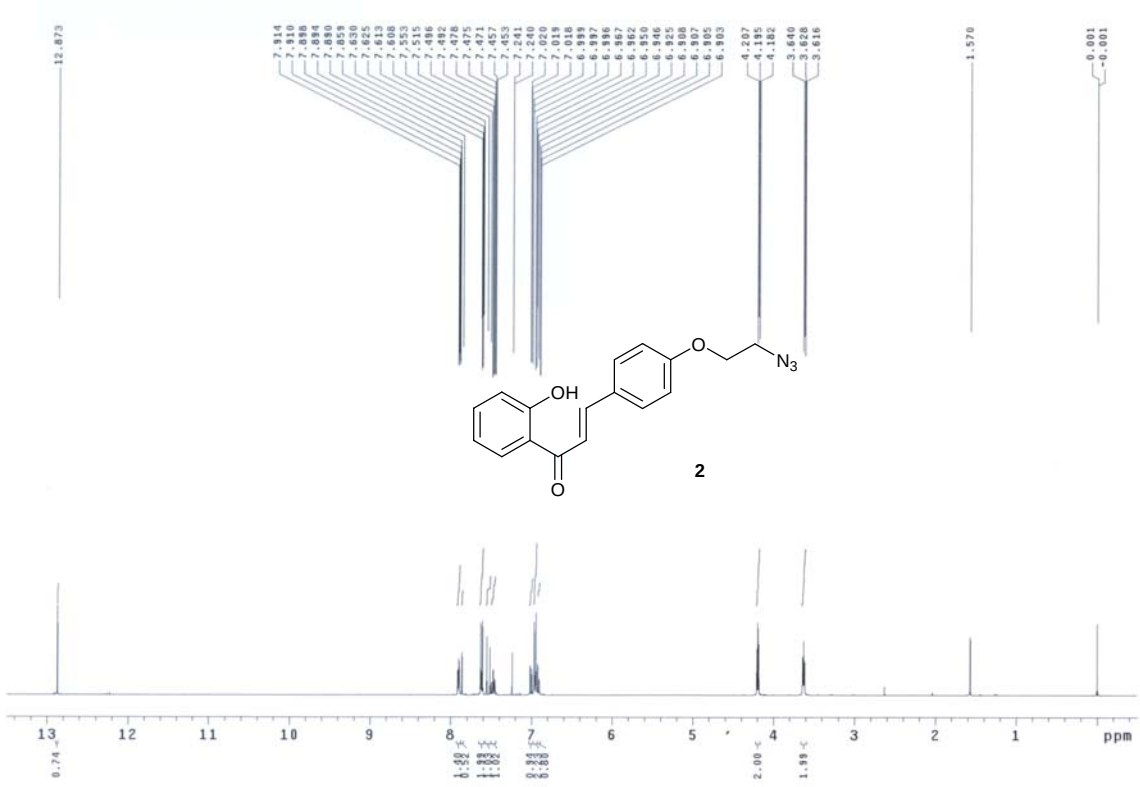


Fig. S7 ^{1}H NMR spectrum (400 MHz) of compound **2** in $CDCl_3$

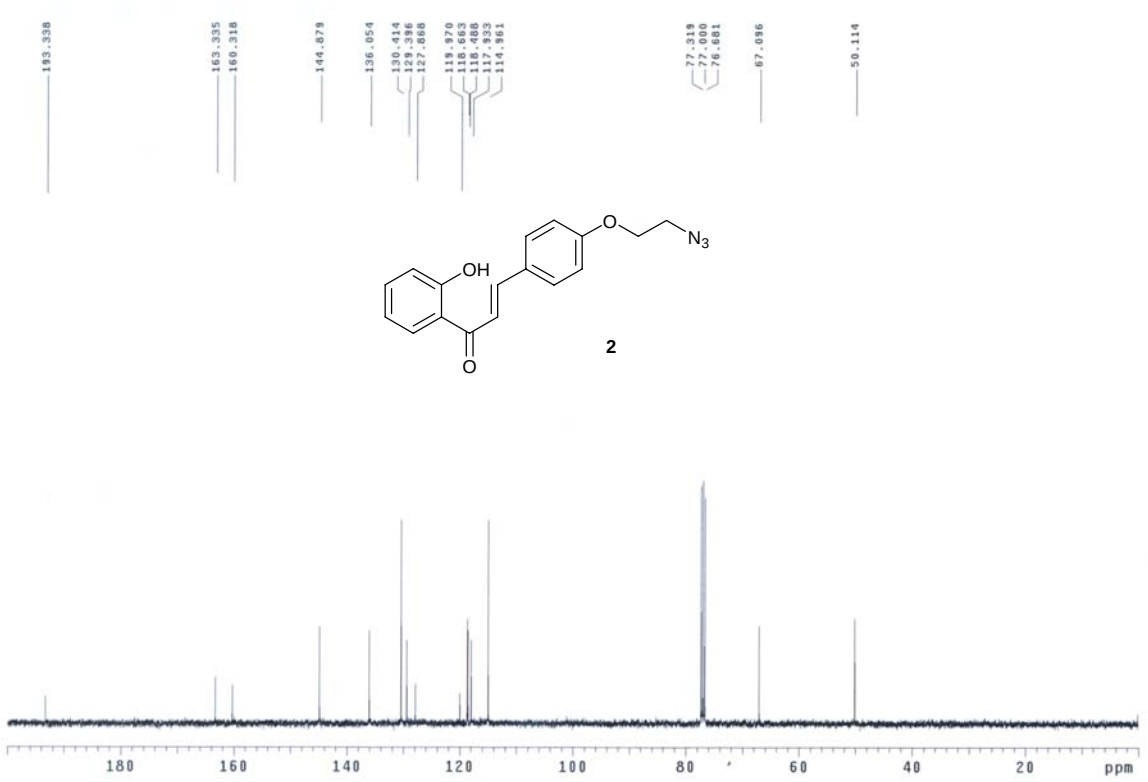


Fig. S8 ^{13}C NMR spectrum (100 MHz) of compound **2** in $CDCl_3$

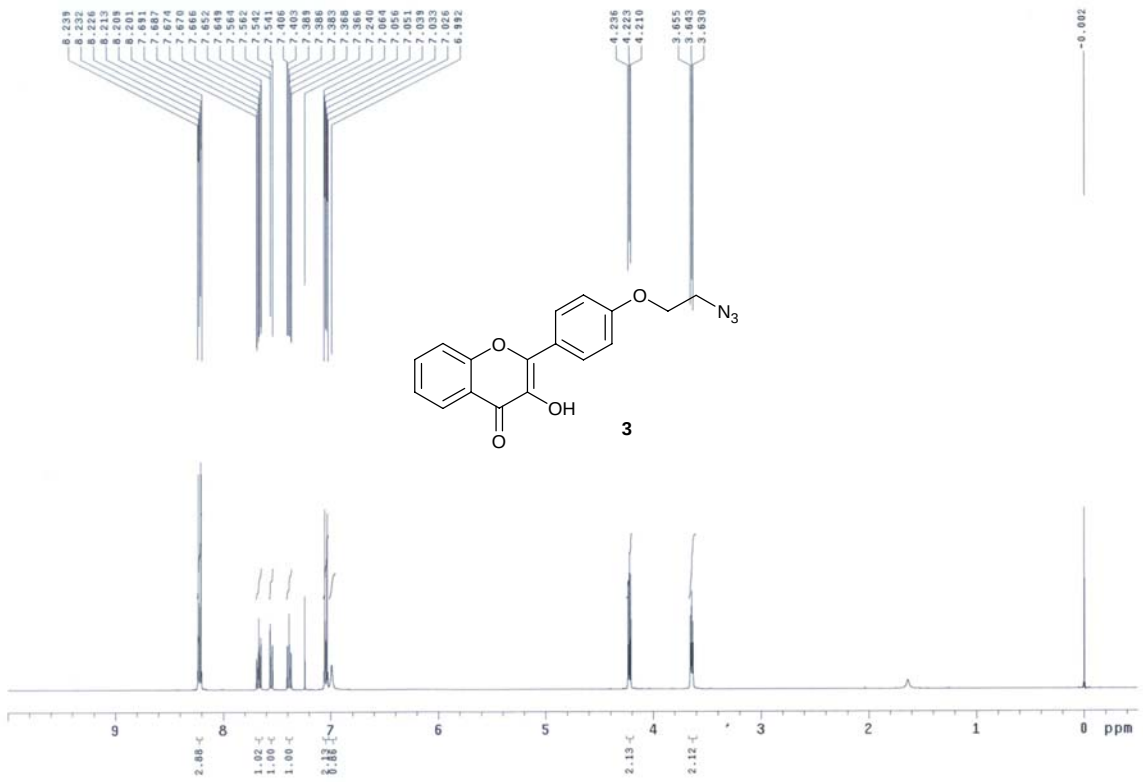


Fig. S9 ^{1}H NMR spectrum (400 MHz) of compound **3** in $CDCl_3$

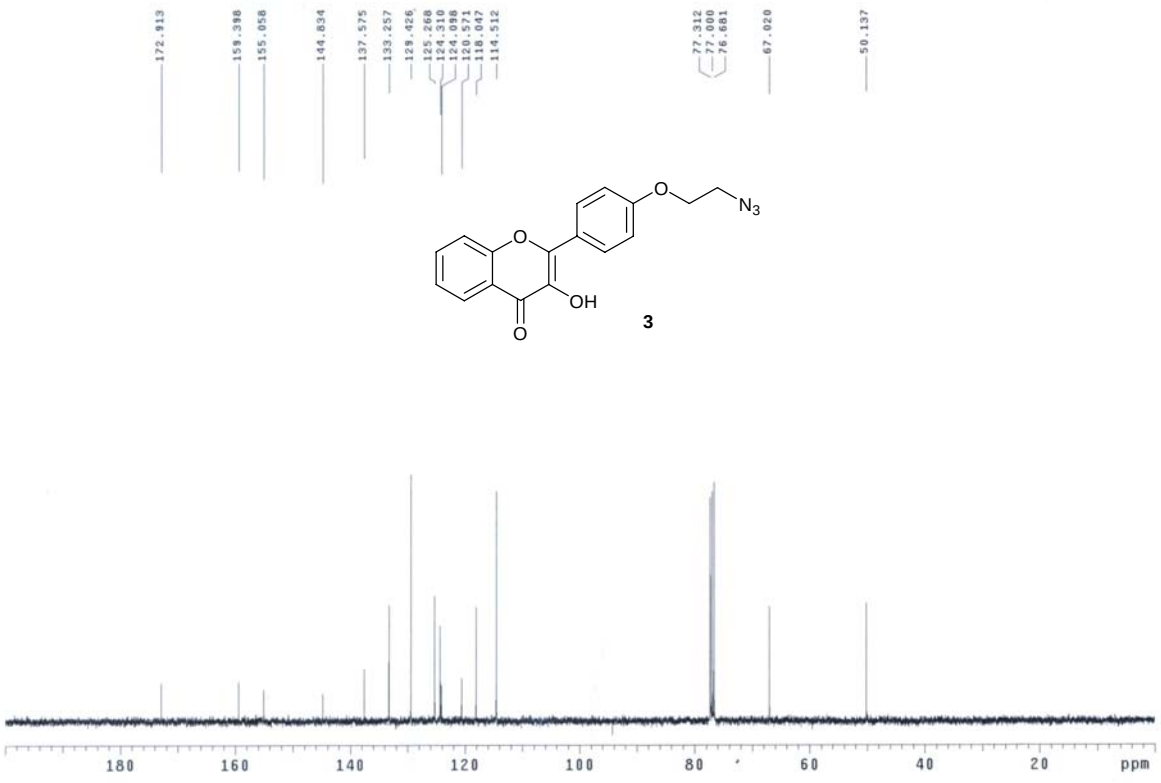


Fig. S10 ^{13}C NMR spectrum (100 MHz) of compound **3** in $CDCl_3$

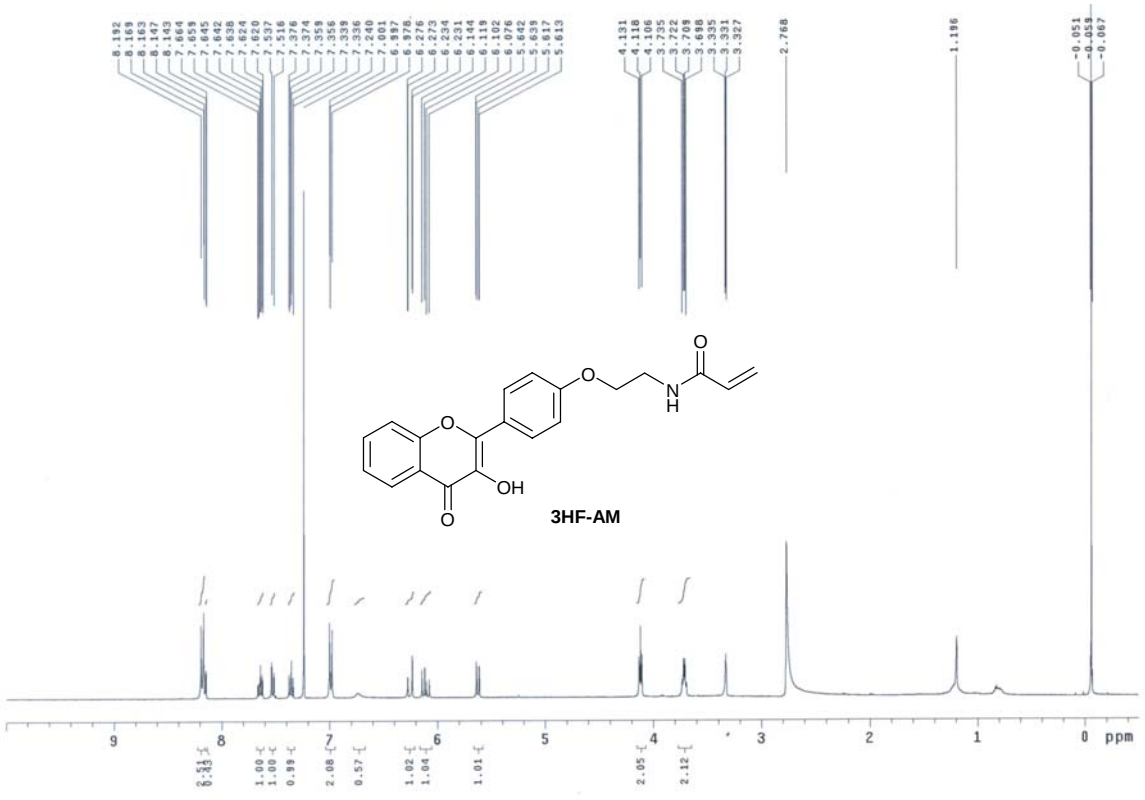


Fig. S11 ^{1}H NMR spectrum (400 MHz) of **3HF-AM** in $CD_3OD/CDCl_3$

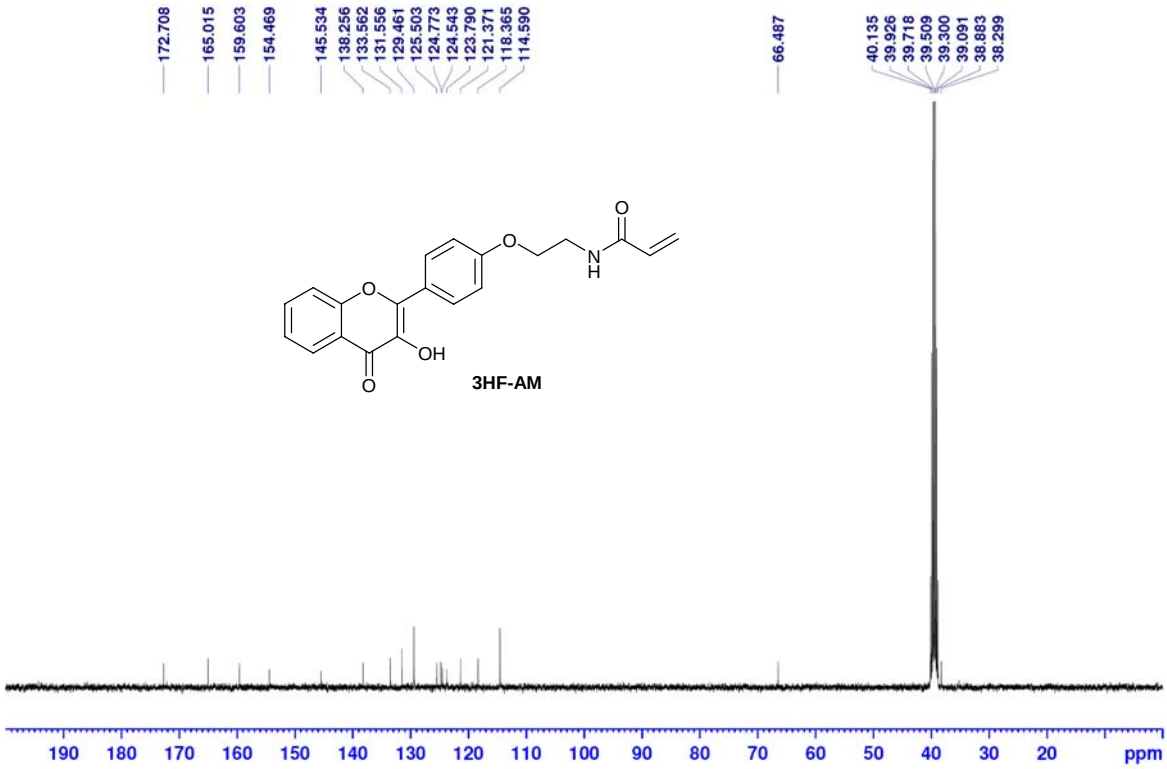


Fig. S12 ^{13}C NMR spectrum (100 MHz) of **3HF-AM** in DMSO-*d6*