

Supporting information

Fluorescence Imaging Lysosome Changes During Cell Division and Apoptosis with a Nile Blue Based Near-infrared Emission

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Materials and methods

All of the solvents used were of analytic grade. **NBM** was dissolved in dimethyl sulfoxide (DMSO) to produce 5 mM stock solutions. Aliquots were then diluted to 1 μ M with a Britton-Robinson buffer solution containing 40 mM acetic acid, phosphoric acid, and boric acid. Slight variations in the pH of the solutions were achieved by adding minimal volumes of NaOH or HCl. $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectra were recorded on a VARIAN INOVA-400 spectrometer. Chemical shifts (δ) were reported as ppm (in CDCl_3 or CD_3SOCD_3), with TMS as the internal standard). Mass spectrometric (MS) data were obtained with HP1100LC/MSD MS and an LC/Q-TOF-MS instruments. Fluorescence measurements were performed on a VAEIAN CARY Eclipse fluorescence spectrophotometer (Serial No. FL0812-M018). Excitation and emission slit widths were modified to adjust the fluorescence intensity to a suitable range. Absorption spectra were measured on a Perkin Elmer Lambda 35 UV/VIS spectrophotometer. All pH measurements were performed using a Model PHS-3C meter calibrated at room temperature ($23 \pm 2^\circ\text{C}$) with standard buffers of pH 9.18 and 6.86.

Synthesis

Synthesis of compound 1. An oven-dried two necked flask equipped with a reflux condenser charged with aniline (432.0 mg, 0.3 mmol) and 4-(2-Chloroethyl) morpholine Hydrochloride (465.0 mg, 0.25 mmol) which were dissolved in ethanol (15.0 mL). The CsCO_3 (810.0 mg, 0.25 mmol) dissolved in 5ml H_2O was added and the mixture was stirred to dissolve completely. Then the mixture was heated to 90°C with stirring for 5 h. After evaporation of the solvent, the compound **1** was obtained as violet oil in 42% yield via column chromatography with silica gel (eluting with n-hexane : ethyl acetate = 1:1). $^1\text{H NMR}$ (400 MHz, CDCl_3): 7.86 (m, 1H, $J = 4.0$ Hz), 7.79 (m, 1H, $J = 4.0$ Hz), 7.46 (m, 2H, $J = 4.0$ Hz), 7.35 (t, 1H, $J = 8.0$ Hz), 7.24 (t, 2H, $J = 8.0$ Hz), 6.58 (d, 1H, $J = 4.0$ Hz), 3.75 (t, 4H, $J = 4.0$ Hz), 3.32 (t, 2H, $J = 4.0$ Hz), 2.81 (t, 2H, $J = 8.0$ Hz), 2.54 (t, 4H, $J = 4.0$ Hz). TOF MS: m/z calcd for $\text{C}_{16}\text{H}_{22}\text{N}_2\text{O}$ $[\text{M}]^+$: 257.1654, found: 257.1661.

Preparation of NBM: Ice bath, 5-(dimethylamino)-2-nitrosophenol (270.4 mg; 1.05 mmol) in ethanol (5.0 mL), compound **1** (199.0 mg; 1.20 mmol) were mixed, and concentrated hydrochloride acid (1.0 mL) were added. Then the mixture was vacuum filtered and the filtrate was refluxed for 4.5 h and monitored by TLC (CH₂Cl₂: CH₃OH = 10:1). The solid was obtained by recrystallization with ethanol. Then, the green solid was dried under vacuum (224.4 mg, 53%) with metallic luster was obtained. ¹H NMR (400 MHz, CD₃OD), δ: 3.33 (m, 8H, *J* = 4 Hz), 3.63(s, 3H), 4.01 (s, 5H), 4.23 (d, 2H, *J* = 4 Hz), 7.00 (d, 1H, *J* = 4 Hz), 7.18 (s, 1H), 7.41 (d, 1H, *J* = 8Hz), 7.87 (m, 1H), 7.96 (t, 2H, *J* = 8Hz), 8.54 (d, 1H, *J* = 8Hz), 8.97 (d, 1H, *J* = 8Hz); ¹³C NMR (100 MHz, CD₃OD), δ: 38.11, 39.98, 52.27, 54.59, 63.65, 93.25, 95.75, 116.66, 123.02, 123.30, 124.15, 129.46, 131.29, 131.64, 132.00, 132.91, 133.13, 148.55, 151.92, 156.80, 157.58ppm; TOF MS: *m/z* calcd for C₂₄H₂₇N₄O₂⁺ [M]⁺: 403.2134, found: 403.2143.

Cell incubation: The mammalian cells MCF-7, Hela, and Raw 264.7 were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). The cells were seeded in 24-well flat-bottomed plates and then incubated for 24 h at 37°C under 5% CO₂. **NBM** (50 nM) was then added to the cells and incubated for another 30 min followed. The cells were washed three times with phosphate-buffered saline (PBS). Fluorescence imaging was performed using an OLYMPUS FV-1000 inverted fluorescence microscope with a 100×objective lens.

Determination of quantum yields: The fluorescence quantum yield of **NBM** was determined according to the method below.

$$\varphi_u = \frac{(\varphi_s)(FA_u)(A_s)(\lambda_{exs})(\eta_u^2)}{(FA_s)(A_u)(\lambda_{exu})(\eta_s^2)}$$

Where φ is fluorescence quantum yield; FA is integrated area under the corrected emission spectra; A is the absorbance at the excitation wavelength; λ_{ex} is the excitation wavelength; η is the refractive index of the solution; the subscripts u and s refer to the unknown and the standard, respectively. We chose fluorescein as standard, which has the fluorescence quantum yield of 0.98 in water.

Lysosome staining in live cells: LysoSensorTM Green DND-189 (1 μ M, Invitrogen) was used to co-stain the cells.

Cytotoxicity experiments: Cell viability measurements were performed by reducing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan crystals using mitochondrial dehydrogenases (Mosmann, 1983). Briefly, MCF-7, HeLa and Hepatic cells were seeded in 96-well microplates (Nunc, Denmark) at a density of 1×10^5 cells/mL in 100 μ L of medium containing 10% FBS. After 24 h of cell attachment, the plates were washed with 100 μ L/well PBS. The cells were then cultured in a medium with 0.5 μ M, 1.0 μ M and 2.0 μ M **NBM** for 12 h. Cells in a culture medium without fluorescent dyes were used as the control. Six replicate wells were used for each control and test concentration. MTT (10 μ L, 5 mg/mL) in PBS was subsequently added to each well. The plates were then incubated at 37 $^{\circ}$ C for 4 h in a 5% CO₂ humidified incubator. The medium was carefully removed, and the purple products were lysed in 200 μ L DMSO. The plate was shaken for 10 min, and the absorbance was measured at 405 and 488 nm using a microplate reader (Thermo Fisher Scientific). Cell viability was expressed as a percentage of the control culture value.

Photodegradation Experiments

In vitro: The photodegradation experiments were performed by placing the sample solutions in square quartz cells (1 \times 1 cm²) and then irradiating the samples with a 500 W I–W lamp at room temperature. To eliminate heat and the absorbance of short-wavelength light, a cold trap [2 L solution of 50 g/L NaNO₂ in 22 cm (height) \times 7 cm (width) \times 22 cm (length)] was set up between the cells and the lamp. The distance between the cells and the lamp was 30 cm. The irreversible bleaching of the dyes at the emission peak was monitored as a function of time. All samples were tightly sealed and not deoxygenated with nitrogen prior to the test to simulate the actual conditions during practical applications (unless specified otherwise).

In vivo: HeLa cells were incubated with **NBM** (2 μ M) via the procedure described in the preceding section. The cells were then washed three times with PBS, exposed to laser illumination ($\lambda_{\text{ex}} = 635$ nm), and then imaged for 1800 sec. Data were obtained from eight real-time image areas; the values observed in each case were averaged.

Temporal tracking of lysosome morphology of apoptotic HeLa cells: HeLa cells were prestained with **NBM** using the procedure described for lysosome staining. The cells were treated with 10 ng mL⁻¹ of TNF- α to induce autophagy. The cells were then analyzed with confocal fluorescence microscope for 60 min.

Live cell division: Hepatic cells was cultured for 3 days in phenol-red free Dulbecco's Modified Eagle's Medium (DMEM, WelGene) supplemented with penicillin/streptomycin and 10% fetal bovine serum (FBS; Gibco) in a CO₂ incubator at 37 °C. One day before imaging, cells were seeded in glass bottom dish (MatTek, 35mm dish with 20 mm bottom well). The next day the live cells were incubated with 1.0 μ M **NBM** and 5.0 μ M Hoechst 33258 for 30 min at 37 °C under 5% CO₂ and then added 100 μ M 6-benzylamino adenine for 150 min at 37 °C under 5% CO₂. After, they were washed with phosphate-buffered saline (PBS) three times.

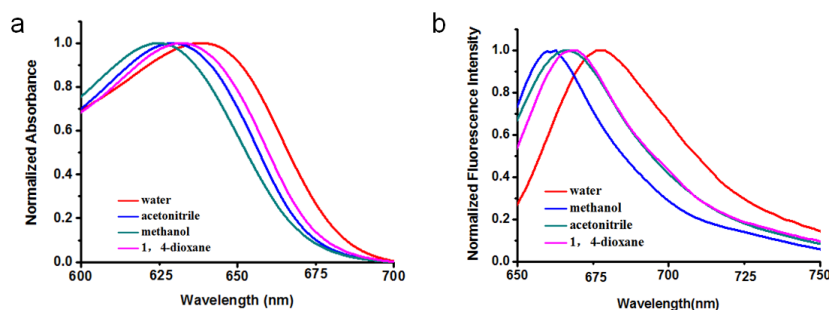


Fig. S1 Normalized absorbance and emission spectra of **NBM** (5 μ M) in different solutions.

Table S1 Photophysical properties of **NBM** in various solvents.

Test System	$\lambda_{\text{abs}}^{\text{[a]}}$	$\lambda_{\text{em}}^{\text{[b]}}$	Stokes shift	$\Phi_{\text{F}}^{\text{[d]}}$	$\epsilon^{\text{max(M}^{-1}\text{.cm}^{-1}\text{)[c]}$
H ₂ O	638 nm	678 nm	40 nm	0.10	2.7×10^4
CH ₃ OH	629 nm	669 nm	40 nm	0.08	5.5×10^4
acetonitrile	626 nm	668 nm	42 nm	0.42	6.2×10^4
1,4-dioxane	624 nm	666 nm	42 nm	0.35	2.9×10^4

Table S2 Fluorescence intensity of **NBM** and LysoTracker Green changes as a function of time irradiated with a 500 W I–W lamp at room temperature.

<i>NBM</i>			<i>LysoTracker Green</i>		
Time (min)	Maximal Fluorescence Intensity (a.u.)	Normalized Intensity	Time (min)	Maximal Fluorescence Intensity (a.u.)	Normalized Intensity
0	632.7	1.000	0	511.9	1.000
30	585.9	0.926	30	397.2	0.776
60	607.4	0.960	60	361.9	0.707
90	599.2	0.947	90	306.6	0.599
120	591.6	0.935	120	262.6	0.513
150	589.0	0.931	150	262.1	0.512
180	570.7	0.902	180	259.5	0.507
210	576.4	0.911	210	266.7	0.521
240	576.4	0.911	240	266.2	0.520

The ionic interference experiment

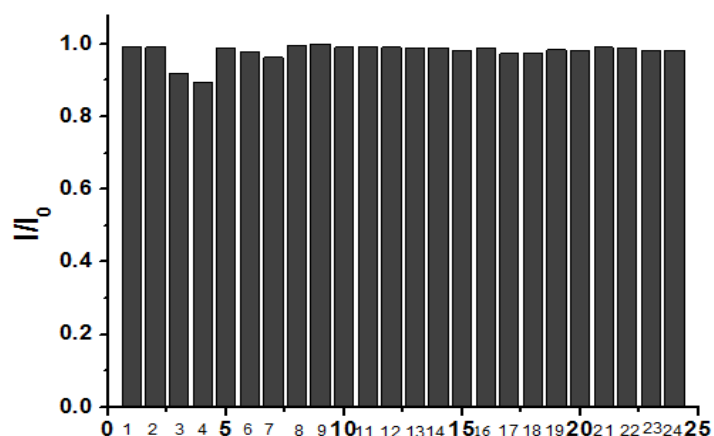


Fig. S2 Fluorescence intensity of **NBM** in the absence or presence of miscellaneous ions in 40 mM HEPES aqueous solution (pH 5.0). From left to right: **NBM**, Zn^{2+} , Ag^+ , Pd^{2+} , Hg^{2+} , Cr^{3+} , Cu^{2+} , Al^{3+} , Fe^{2+} , Fe^{3+} , Cd^{2+} , Mg^{2+} , NH_4^+ , Mn^{2+} , K^+ , Na^+ , Co^{2+} , Ni^{2+} , Ca^{2+} , ClO_4^- , NO_3^- , Cl^- , I^- , Br^- , S^{2-} . Condition: excitation wavelength is 639 nm, emission wavelength is 678 nm. 5 μM for **NBM**; 50 μM for all the ions.

The fluorescence image of NSCs cell staining with NBM

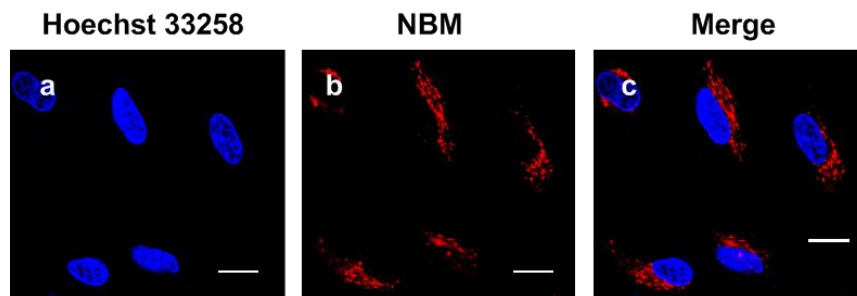


Fig. S3 Confocal fluorescence images of **NBM** (50 nM) in NSCs cells co-stained with nuclei staining Hoechst 33258 (5 μM). a: red emission from **NBM**. b: blue emission from Hoechst 33258. c: overlay of the blue and red channels. Scale bars represent 10 μm .

Photostability in solution and cell

Photobleaching is a common problem for many organic dyes, with the reporting dyes often compromising the temporal monitoring of dynamic events inside cells.^{s1} In this study, under irradiation from a 500 W *I-W* lamp for 4 h, the maximal fluorescence intensity of **NBM** remained nearly constant (Fig. S4 and Table S2). In addition, when mice hippocampal neurons cells prestained with **NBM** and LysoTracker Green were exposed to constant laser illumination for 30 min, the fluorescence of LysoTracker Green quickly decayed in 5 min (Fig. S5b) while **NBM** exhibited constant fluorescence emission (Fig. S5a). These experimental results indicated the high photostability of **NBM** under environmental and biological conditions.

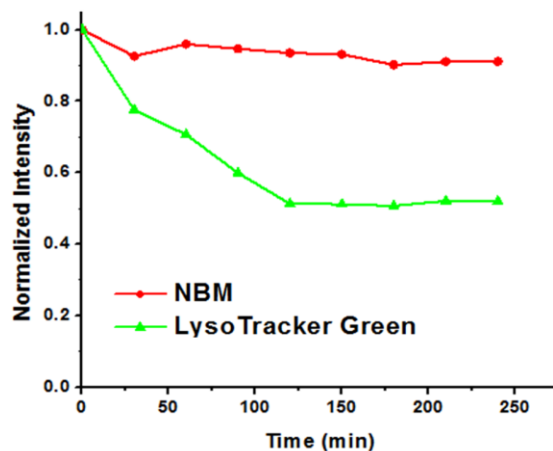


Fig. S4 Comparisons on the photofading of **NBM** ($5 \mu\text{M}$) and LysoTracker Green ($5 \mu\text{M}$) in HEPES buffer (40 mM , $\text{pH}=7.4$). **NBM** and LysoTracker Green were excited at 638 nm and 504 nm , respectively.

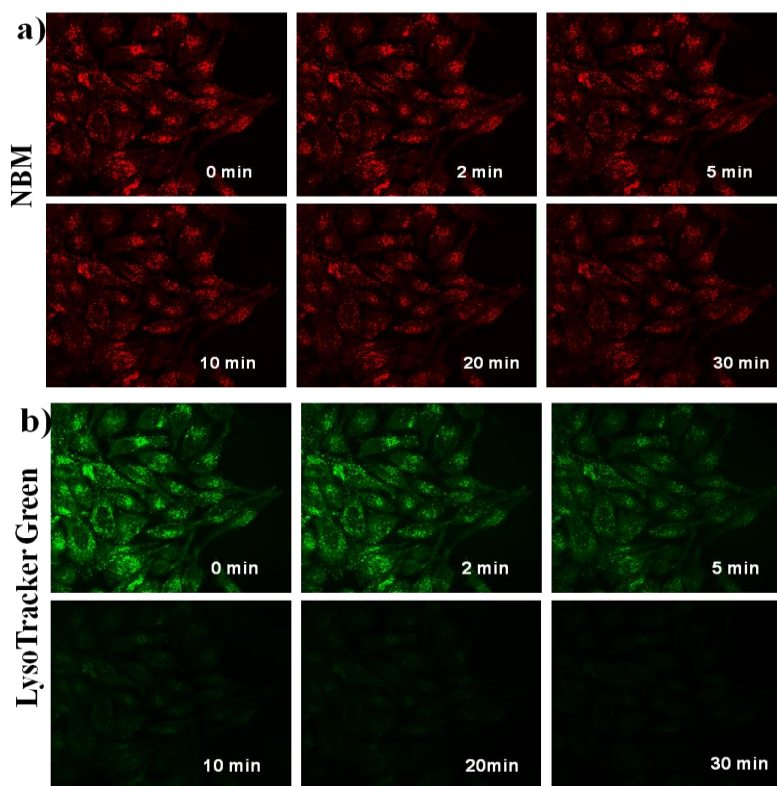


Fig. S5 Photostability of **NBM** and LysoTracker Green in live NSCs cells. Cells were stained with: a) $1 \mu\text{M}$ **NBM**, b) $1 \mu\text{M}$ LysoTracker Green. **NBM** and LysoTracker Green were excited at 635 nm and 488 nm , respectively. The fluorescence images were recorded at $670\text{-}710 \text{ nm}$ (**NBM**) and $495\text{-}515 \text{ nm}$ (LysoTracker Green), respectively.

Cytotoxicity by MTT method.

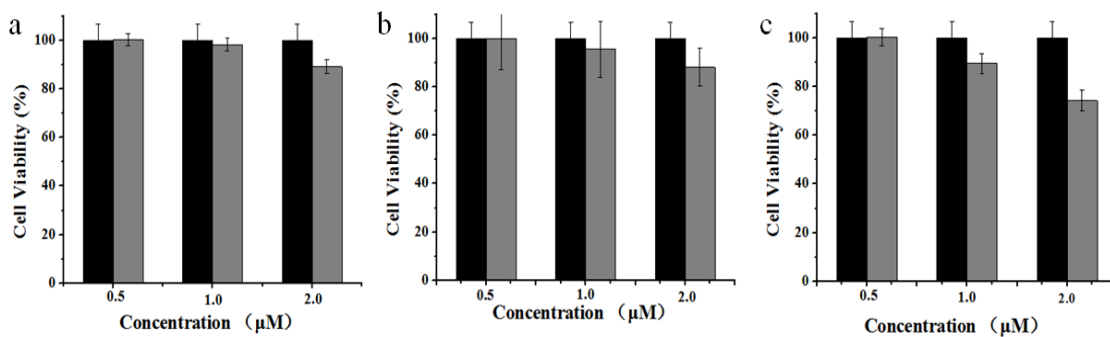


Fig. S6 Comparisons on the cytotoxicity of NBM and LysoTracker Green with various concentrations (0.5 μM , 1.0 μM and 2.0 μM) in living MCF-7 (a), HeLa (b) and Hepatic cells (c) for 12 h.

The fluorescence image of MCF-7, HeLa and HepG-2 cells stained with NBM (25 nM)

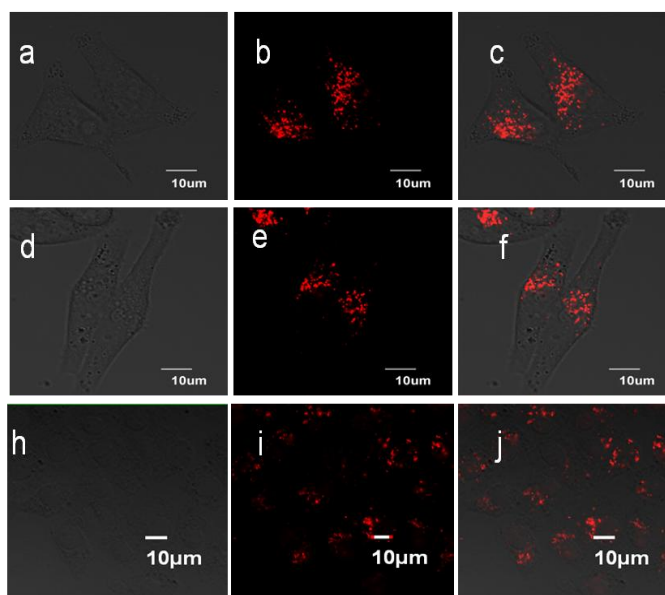


Fig. S7 Fluorescence images of NBM (25 nM) in MCF-7 (a-c), HeLa (d-f) and HepG-2 (h-j) at 37°C for 30 min. The excitation wavelengths were 635 nm, the fluorescence was collected at 670-710 nm. Scale bars represent 10 μm .

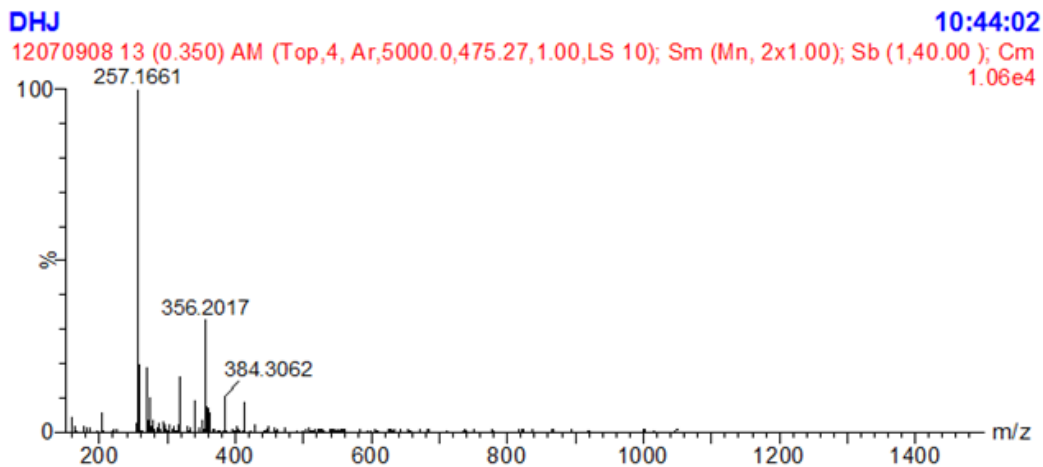


Fig. S8 TOF mass of compound **1**.

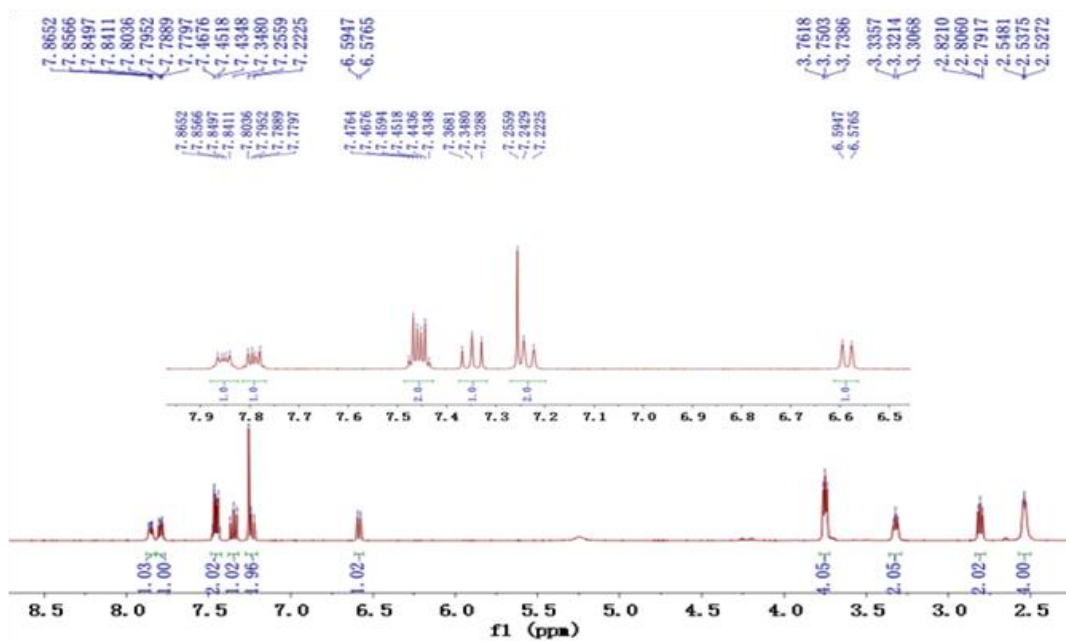


Fig. S9 $^1\text{H-NMR}$ spectrum of compound **1** recorded in CDCl_3 .

DHJ-1

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12103000 54 (1.369) AM (Top, 4, Ar, 5000.0, 475.27, 1.00, LS 10); Sm (Mn, 2x1.00); Sb (1, 40.00); Cm (45:59)

1.01e4

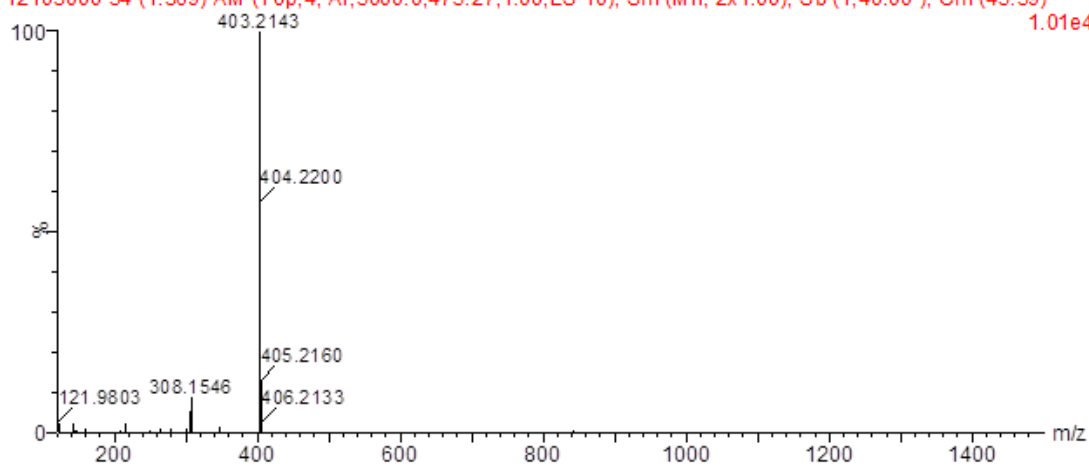


Fig. S10 TOF mass of compound NBM.

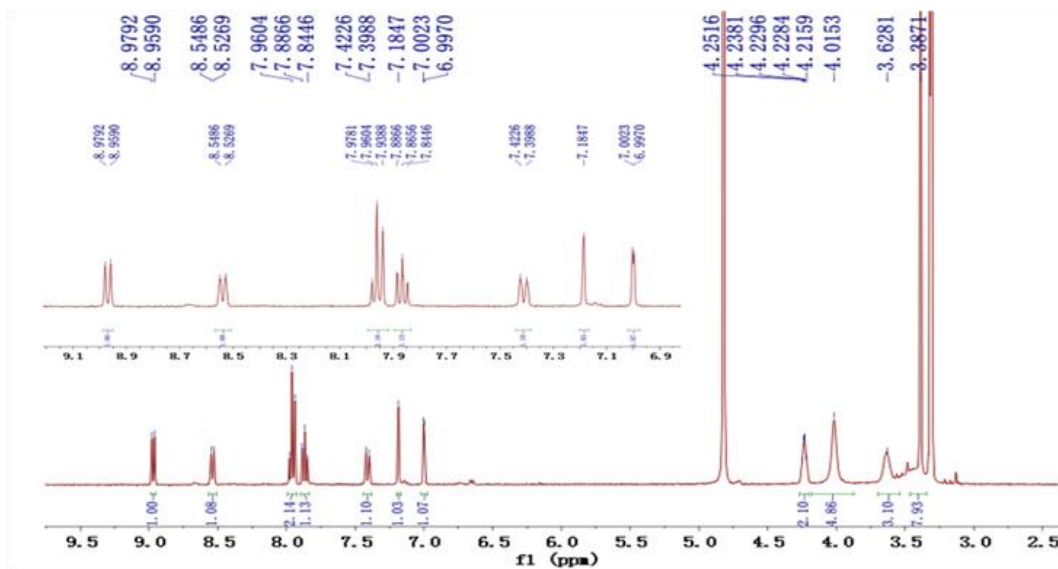


Fig. S11 $^1\text{H-NMR}$ spectrum of NBM recorded in CD_3OD .

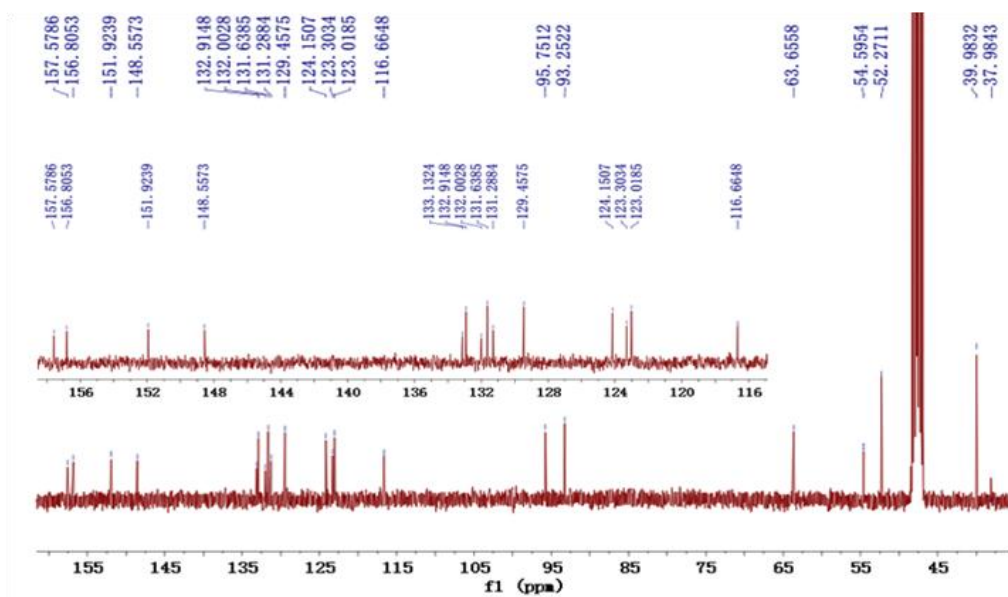


Fig. S12 ^{13}C -NMR spectrum of NBM recorded in CD_3OD .

References

S1. Z. Li, S. Wu, J. Han and S. Han. *Analyst.*, 2011, **136**, 3698-3706;