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Supplementary Material

Diaminomaleonitrile-functionalised Schiff Bases: Synthesis, Solvatochromism, and Lysosome-specific Imaging

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Materials and General Information

All starting materials were commercially available. Reagents and solvents were purchased from Sigma-Aldrich, Accela Chembio, AK Scientific, and Chem-Supply, and used as received without purification.

^{1}H-NMR and ^{13}C-NMR spectra were recorded using 400 MHz Bruker AV3HD-400 spectrometer with tetramethylsilane (TMS, $\delta = 0$) as the internal standard. HRMS spectra were acquired using a Thermo Scientific Q Exactive Plus Orbitrap LC-MS/MS instrument operating in ESI (Electrospray ionization) mode. UV-Visible absorption spectra were recorded at room temperature on an Agilent Cary 300 UV-visible spectrophotometer equipped with a 1.0 cm quartz cell. Fluorescence emission spectra were recorded on an Agilent Cary Eclipse Fluorescence Spectrophotometer and used 1.0 cm quartz cells. Data were plotted by using Origin 2019.

For UV-Vis absorption measurement, the background of (co-)solvent alone was subtracted. For fluorescence measurement, the emission slit was set at 10 nm, and the scan speed was set at medium. The concentrations of five compounds in photophysical tests were 10 μM.

All stock solutions of compounds were prepared at 1 mM in DMSO for solvatochromism and in THF for AIE curve measurements. And the pH differentiation property of Lyso-BAM was validated by diluting the 1 mM DMSO stock solution in buffers with pH values ranged from 4 to 9. Briefly, buffers of pH 4 and 5 were prepared from the 0.05 M AcONa/AcOH aqueous solution; buffers of pH 6, 7 and 8 were prepared from the 0.05 M K_2HPO_4/KH_2PO_4 aqueous solution; buffers of pH 9 was prepared from the 0.05 M $NaHCO_3/Na_2CO_3$ aqueous solution. The pH values of mentioned buffers were well quantified by a pH meter before being used.

Cell culture and Imaging

pCDH-EF1-mApple-Lysosomes-20-IRES-Puro constructed with the mApple-Lysosomes-20 sequence available (Addgene plasmid: 54921), which was cut and pasted into a purchased lentivirus expressing vector pCDH-EF1-MCS-IRES-Puro (System Biosciences).

A549-Lysosome20-mApple cell line was stably transducted with lentivirus expressing pCDH-EF1-mApple-Lysosomes-20-IRES-Puro vector. Cells then were maintained in Dulbecco Modified Eagle Medium (Gibco)

supplied with 10% fetal bovine serum (FBS) and Penicillin-Streptomycin (100 U/mL) supplying with 2 μg/mL puromycin for selection.

FACS sorting was conducted using a BD FACSAria III, with gating as all live/singlet/mApple^{pos} cells. Cell purity was checked postsort. Sorted cells were then cultured in DMEM with 10% FBS and Penicillin-Streptomycin (100 U/mL) in a 5% CO_2 humidity incubator at 37 ℃.

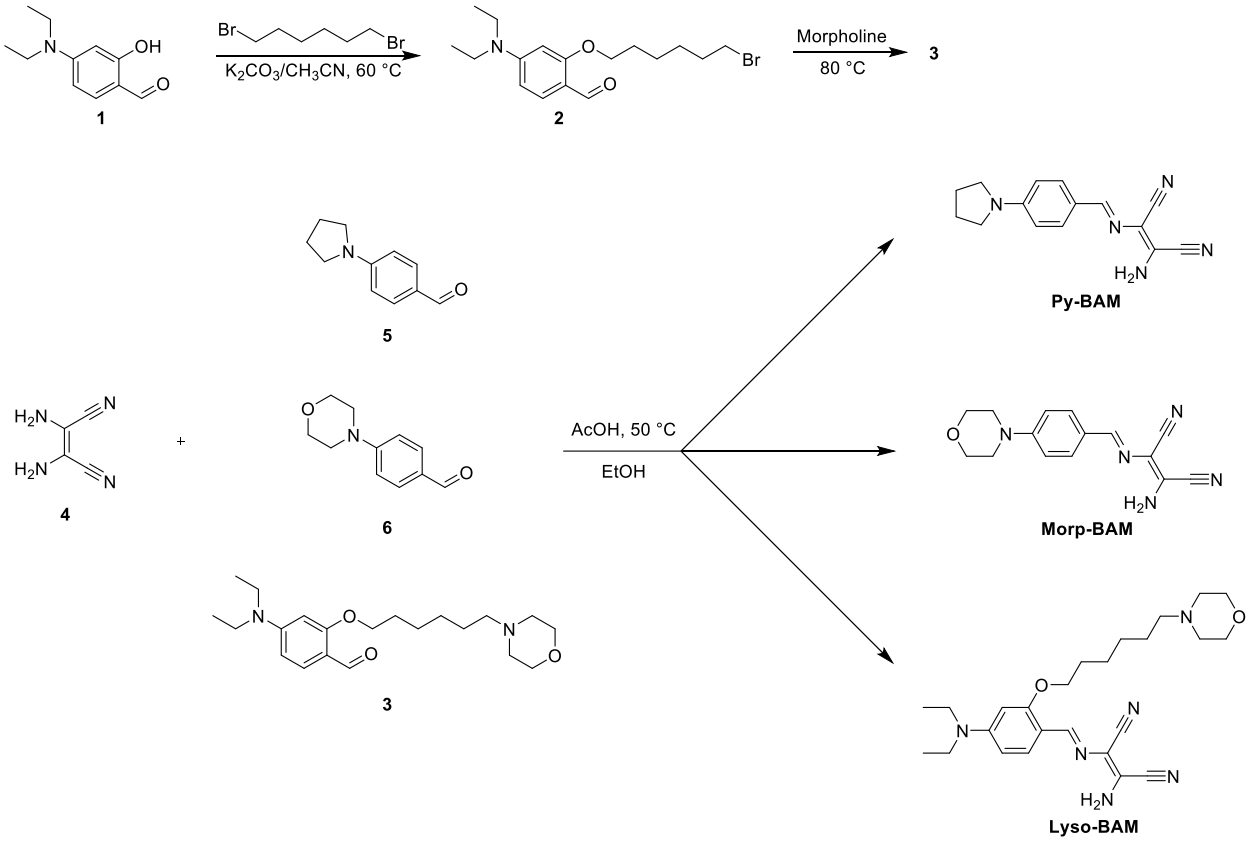
Lyso-BAM was stored as stock solution at 2.5 mM (100×) in DMSO.

For the cell viability assay, seeded A549 cells were cultured overnight to reach approximately 80 % confluency. Then the cells were rinsed with PBS and stained with 10 μM, 25 μM or 50 μM of freshly diluted Lyso-BAM in DMEM at 37 °C for 40 min. Cells were also treated with corresponding amount of DMSO to exclude the cytotoxicity of DMSO. Afterwards cells were rinsed with PBS again and incubated with 10 % AlamarBlue™ reagent in complete DMEM at 37 °C for 4 h in darkness. The AlamarBlue™ reagent was excited at the wavelength of 540–570 nm, and emission was recorded at 580–610 nm by plate reader. Positive controls (by killing cells with 70 % ethanol) and negative controls (cultured in complete DMEM without any treatment) were properly set. Cytotoxicity was calculated by the following formula: Normalized cell viability = (sample RFU−positive control RFU)/(negative control RFU−positive control RFU), where RFU represents “relative fluorescence units”.

Cells were directly seeded on pre-sterilized Ibidi μ-Slide 8-well chambers at a density of 2×10^4 cells per well and incubated in cell culture medium for 24 h prior to staining. Afterwards, cells were treated with 25 μM of Lyso-BAM at 37 ℃ for 40 min and excess dye was washed away with phosphate buffered saline (PBS). The cells were subsequently fixed in 4% (w/v) paraformaldehyde (PFA) solution in PBS. The stained and fixed cells were then rinsed with PBS and maintained in cold PBS for imaging.

Imaging was performed on chambers using the Zeiss LSM 800 confocal microscope for Lyso-BAM (excitation: 488 nm, 0.3% laser power; emission: 450-550 nm) and mApple-fused LAMP1 (excitation: 561 nm, 0.15% laser power; emission: 571-650 nm) using a 63× objective lens.

Synthesis and Characterization



Scheme S1. Synthetic route of Py-BAM, Morp-BAM and Lyso-BAM.

2-((6-bromohexyl)oxy)-4-(diethylamino)benzaldehyde (**2**). The synthetic route towards **2** was adopted from the strategy previously described by Shi et al[1]. To a stirred solution of 4-(diethylamino)salicylaldehyde (1.93 g, 10 mmol) and K_2CO_3 (1.66 g, 12 mmol) in acetonitrile was added 1,6-dibromohexane (1.54 mL, 10 mmol) dropwise via a syringe at room temperature. The resulting mixture was heated at 60 ℃ under nitrogen for overnight. After cooling to room temperature, the reaction mixture was evaporated under reduced pressure and extracted with DCM. The combined organic fraction was dried using anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was further purified by silica gel column chromatography using petroleum spirits and EA (1:1, v/v) as eluant to afford compound **2** as a colourless oil (2.67 g, yield 75%). ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 10.02 (s, 1H), 7.49 (d, J = 8.9 Hz, 1H), 6.33 (dd, J = 8.9, 1.8 Hz, 1H), 6.13 (d, J = 2.2 Hz, 1H), 5.91 – 5.66 (m, 1H), 4.08 (t, J = 6.3 Hz, 2H), 3.53 (t, J = 6.7 Hz, 2H), 3.43 (q, J = 7.1 Hz, 4H), 1.88 – 1.79 (m, 2H), 1.76 (t, J = 6.4 Hz, 2H), 1.46 (dd, J = 7.3, 3.6 Hz, 4H), 1.12 (t, J = 7.0 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6), δ (ppm): 185.7, 163.7, 154.1, 129.9, 113.8, 104.7, 93.9, 68.0, 55.4, 44.5, 35.5, 32.6, 28.8, 27.7, 25.2, 12.9.

4-(diethylamino)-2-((6-morpholinohexyl)oxy)benzaldehyde (**3**). The synthetic route towards **3** was adopted from the strategy previously described by Leung et al[2]. Compound **2** (2.14g, 6 mmol) was added into morpholine (20 mL), and the mixture was heated at 80 ℃ under nitrogen for 8 h. Then the mixture was concentrated under vacuum and extracted with DCM for three times. The extracts were washed with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was further separated by silica gel column chromatography using methanol/DCM (1:20, v/v) as the eluting solvent to give a pale yellow solid (1.96 g, yield 90%). ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 10.02 (s, 1H), 7.49 (d, J = 9.0 Hz, 1H), 6.33 (dd, J = 9.2, 1.6 Hz, 1H), 6.13 (d, J = 2.3 Hz, 1H), 4.08 (t, J = 6.3 Hz, 1H), 3.57 – 3.51 (m, 1H), 3.43 (q, J = 7.1 Hz, 1H), 2.30 (s, 1H), 2.27 – 2.21 (m, 1H), 1.80 – 1.71 (m, 1H), 1.45 (d, J = 6.0 Hz, 1H), 1.34 (d, J = 6.9 Hz, 1H), 1.12 (t, J = 7.0 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6), δ (ppm): 185.6, 163.8, 154.1, 129.8, 113.8, 104.7, 93.9, 68.1,

66.7, 58.7, 53.9, 44.5, 29.0, 27.0, 26.3, 26.0, 12.9. HRMS (ESI-MS): m/z 363.2638, calcd. 362.2569.

The general synthetic method of diaminomaleonitrile-functionalized Schiff bases was adopted from the strategy previously described by Han et al[3]. Briefly, benzaldehyde was added into a 25 mL Schlenk line and dissolved with a small amount of THF. Subsequently, equivalent molarity of diaminomaleonitrile in ethanol and 2-3 drops of acetic acid was added, and the mixture was heated up to 50 ℃ and stirred for about 60 h. After cooling to room temperature, the resulting solid was filtered off, washed several times with ice-cold ethanol and dried over to afford yellow or orange powders as final products.

Me-BAM (yield 62%). ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 8.10 (s, 1H), 7.82 (d, J = 8.9 Hz, 2H), 7.45 (s, 2H), 6.74 (d, J = 8.9 Hz, 2H), 3.02 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$), δ (ppm): 160.7, 160.6, 157.8, 136.1, 129.3, 128.2, 120.3, 119.3, 116.7, 109.6.

Et-BAM (yield 72%). ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 8.08 (s, 1H), 7.79 (d, J = 9.0 Hz, 2H), 7.40 (s, 2H), 6.70 (d, J = 9.1 Hz, 2H), 3.46 – 3.37 (m, 4H), 1.12 (t, J = 7.0 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6), δ (ppm): 155.9, 150.6, 131.7, 124.3, 122.8, 115.5, 114.6, 111.4, 104.9, 44.3, 12.9.

Py-BAM (yield 67%). ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 8.09 (s, 1H), 7.81 (d, J = 8.8 Hz, 2H), 7.40 (s, 2H), 6.58 (d, J = 8.9 Hz, 2H), 3.42 – 3.24 (m, 7H), 2.02 – 1.88 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6), δ (ppm): 156.0, 150.5, 131.5, 124.3, 123.0, 115.6, 114.6, 112.0, 104.9, 47.8, 25.4. HRMS (ESI-MS): m/z 266.1400, calcd. 265.1327.

Morp-BAM (yield 72%). ^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 8.14 (s, 1H), 7.87 (d, J = 9.0 Hz, 2H), 7.59 (s, 2H), 6.99 (d, J = 9.0 Hz, 2H), 3.85 – 3.65 (m, 4H), 3.30 – 3.26 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6), δ (ppm): 155.5, 153.7, 131.1, 126.1, 125.4, 115.3, 114.4, 114.2, 104.3, 66.4, 47.5. HRMS (ESI-MS): m/z 282.1342, calcd. 281.1277.

Lyso-BAM (yield 66%). ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 8.72 (s, 1H), 7.82 (d, J = 9.0 Hz, 1H), 6.28 (dd, J = 9.0, 2.1 Hz, 1H), 6.03 (d, J = 2.1 Hz, 1H), 5.01 (s, 1H), 4.00 (t, J = 6.1 Hz, 1H), 3.85 – 3.75 (m, 1H), 3.41 (q, J = 7.1 Hz, 1H), 2.67 (s, 1H), 2.58 – 2.42 (m, 1H), 1.89 – 1.78 (m, 1H), 1.63 (dd, J = 15.5, 7.7 Hz, 1H), 1.53 (dd, J = 15.5, 7.7 Hz, 1H), 1.41 (dd, J = 14.7, 7.6 Hz, 1H), 1.26 – 1.14 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6), δ (ppm): 172.5, 161.3, 152.4, 151.3, 129.7, 123.4, 115.7, 114.6, 111.9, 106.2, 105.2, 94.6, 68.1, 66.7, 58.6, 53.8, 44.5, 29.1, 27.1, 26.4, 26.0, 21.5, 13.0. HRMS (ESI-MS): m/z 453.2974, calcd. 452.2900.

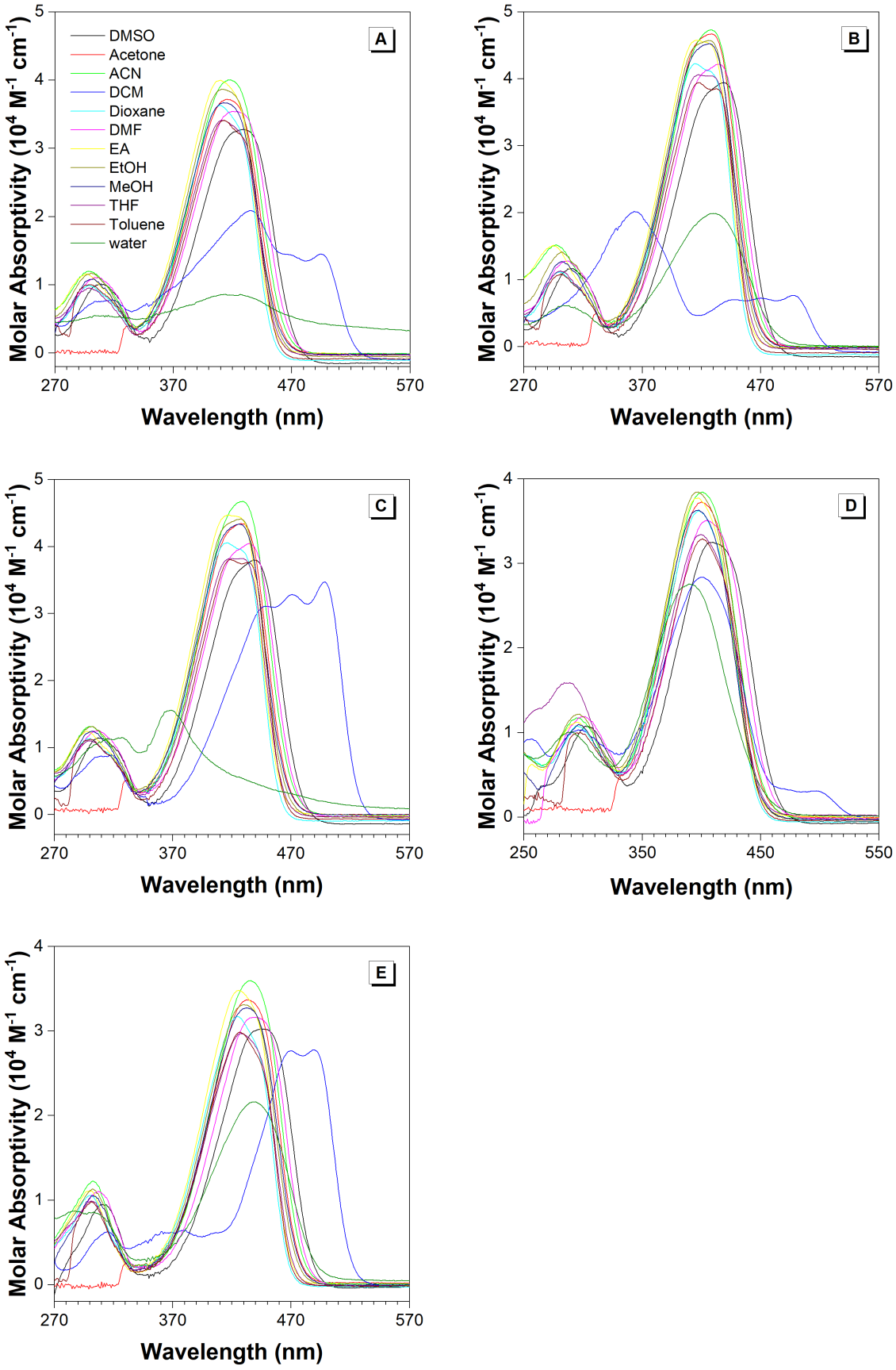


Figure S1. UV-vis absorption spectra of (A) Me-BAM, (B) Et-BAM, (C) Py-BAM, (D) Morp-BAM, and (E) Lyso-BAM in different solvents.

Table S1. Excitation wavelengths (λ_{ex}) of BAMs in different solvents.

Solvent	Me-BAM	Et-BAM	Py-BAM	Morp-BAM	Lyso-BAM
	λ_{ex} (nm)				
Toluene	413	417	419	401	427
Dioxane	409	415	416	398	425
Ethyl acetate	410	416	418	397	425
THF	411	418	421	399	427
DCM	436	363	498	402	490
DMSO	429	438	438	409	444
DMF	424	435	434	405	440
Acetone	415	428	428	400	432
Ethanol	411	426	427	397	431
Acetonitrile	418	429	429	401	436
Methanol	412	426	426	397	432
Water	414	432	369	390	440

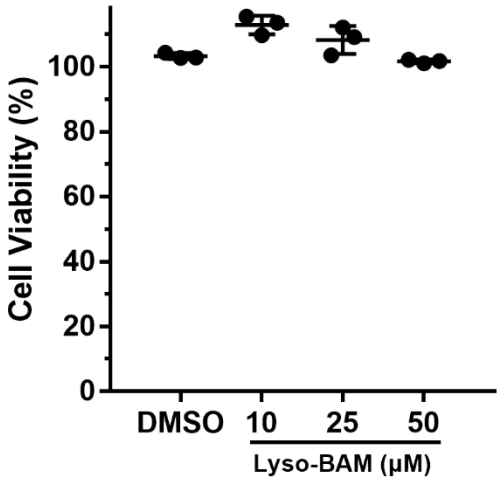


Figure S2. Cell viability assay of Lyso-BAM (n = 3 biological replicates; mean ± s.e.m.). A549 cells were incubated with 10, 25, or 50 μM Lyso-BAM for 40 min. Cells were also treated with corresponding amount of DMSO to exclude the cytotoxicity of DMSO.

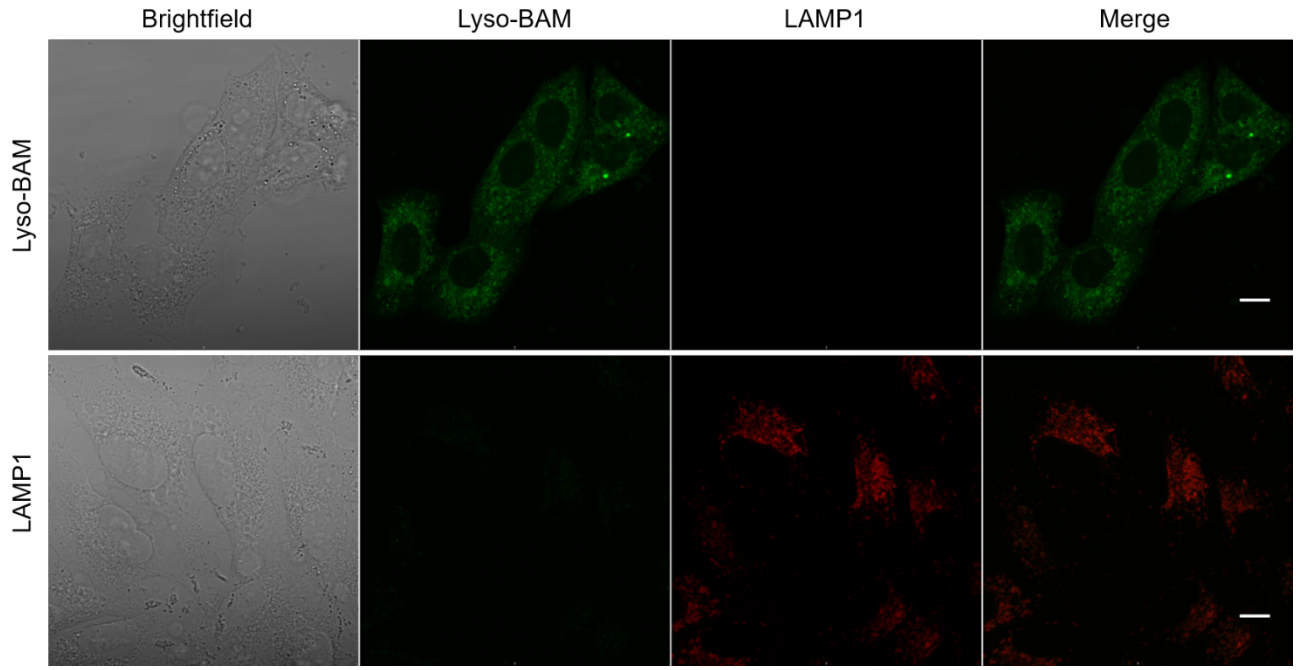


Figure S3. Examination of bleed-through by confocal microscopy. The four columns show signal in the channel of brightfield, Lyso-BAM (excited at 488 nm), mApple (excited at 561 nm), and fluorescence merge images, respectively. Results confirmed that there was neither bleed-through nor autofluorescence under this setting. Scale bar = 10 μm.

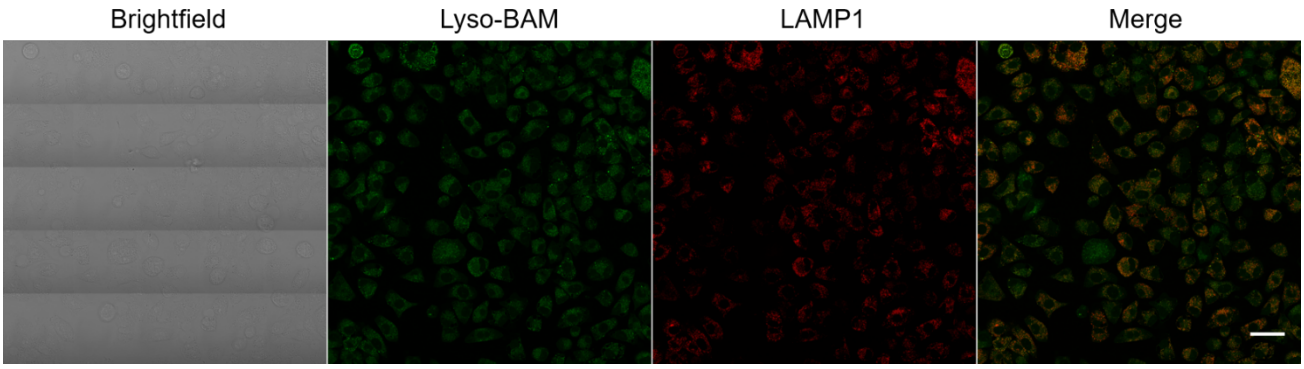


Figure S4. Tile scan image of Lyso-BAM stained A549-Lysosome20-mApple cells. Scale bar = 50 μm.

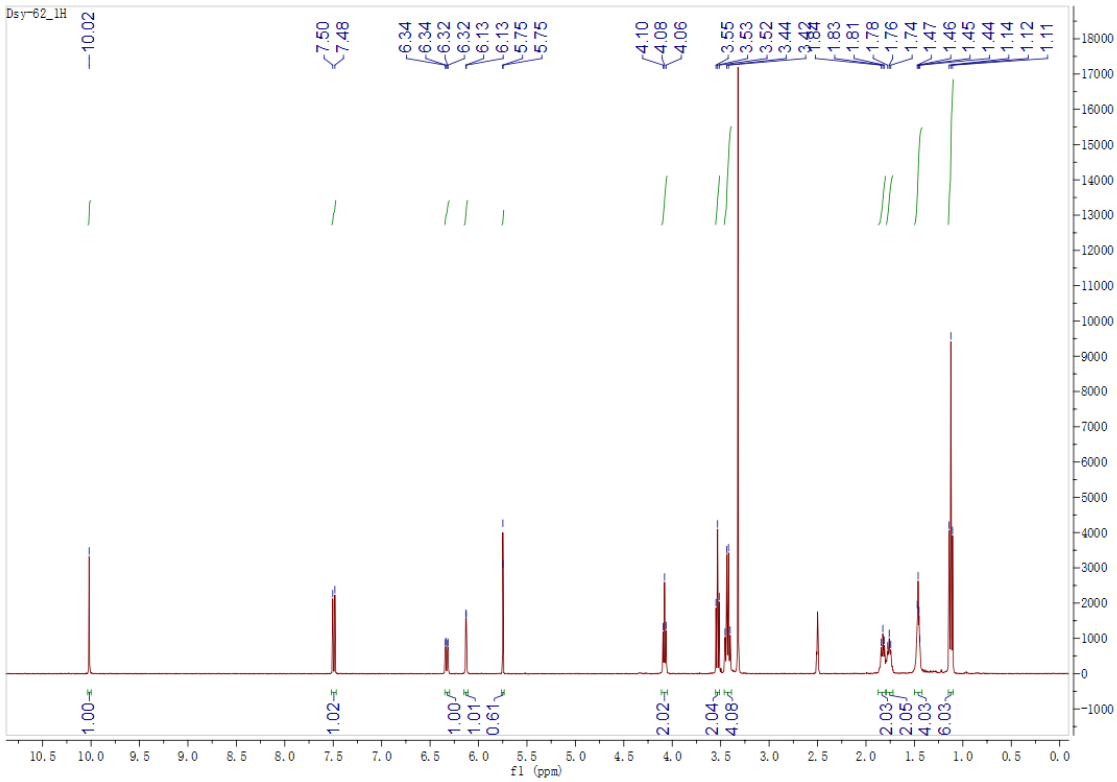


Figure S5. ^{1}H NMR spectrum of compound **2** in DMSO-*d6*.

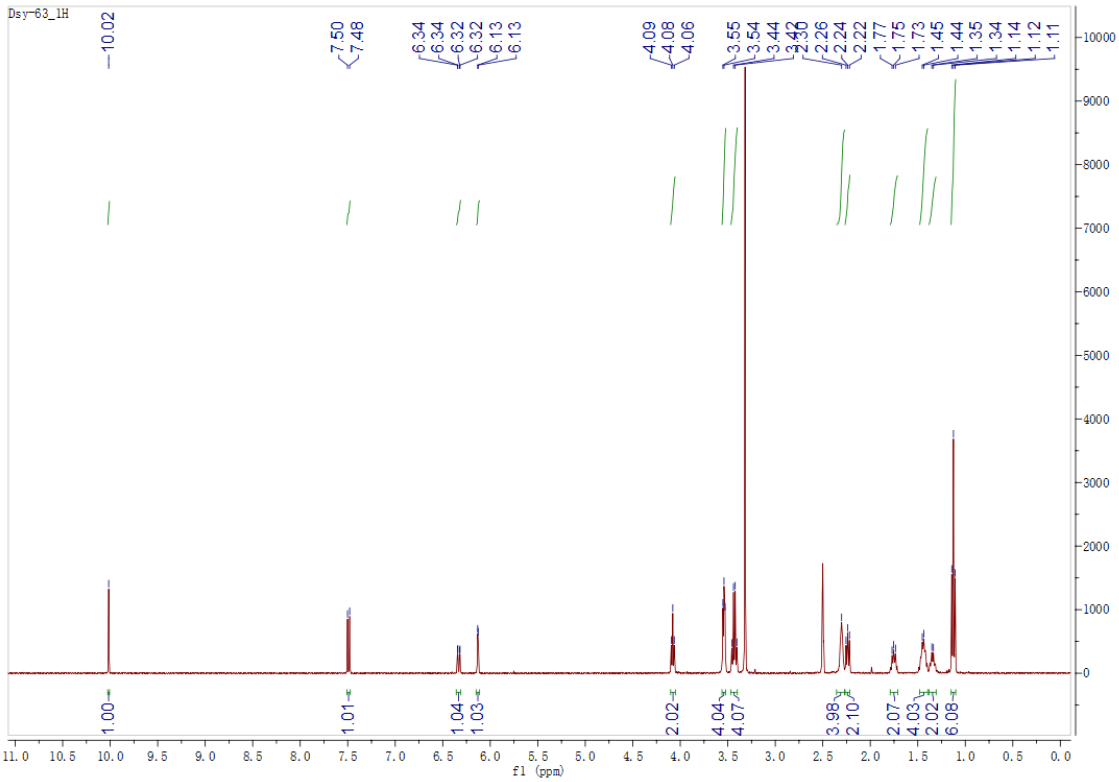


Figure S6. ^{1}H NMR spectrum of compound **3** in DMSO-*d6*.

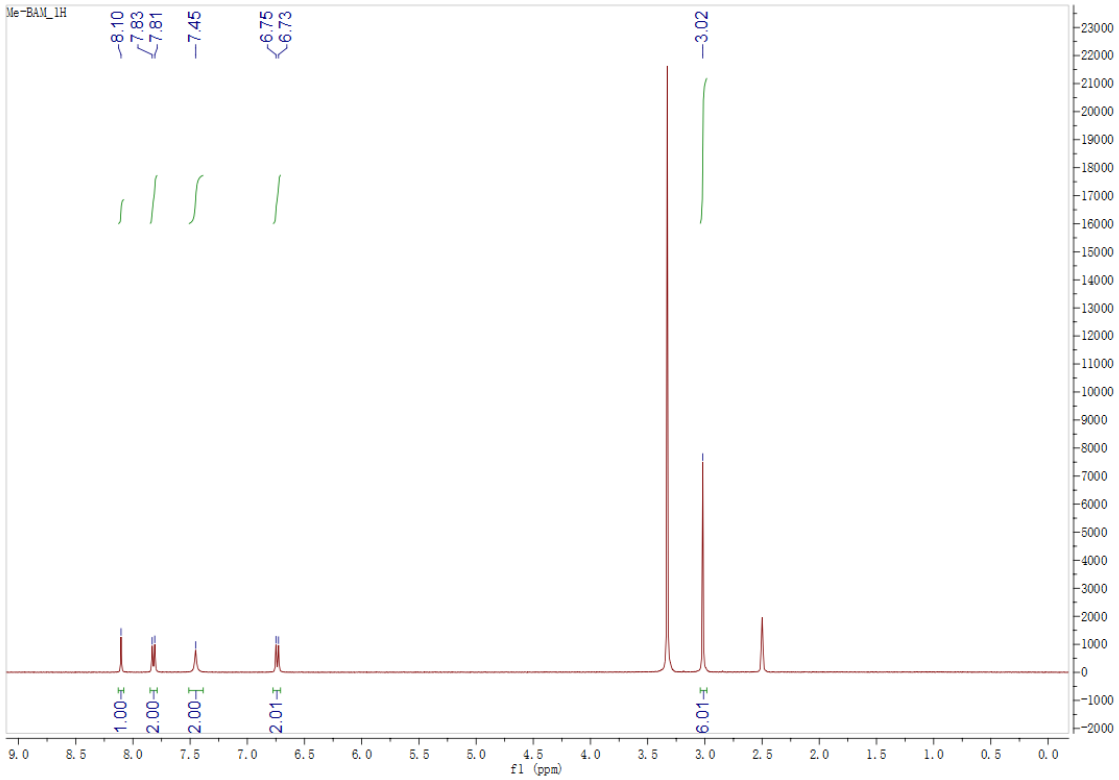


Figure S7. [1]H NMR spectrum of compound Me-BAM in DMSO-*d6*.

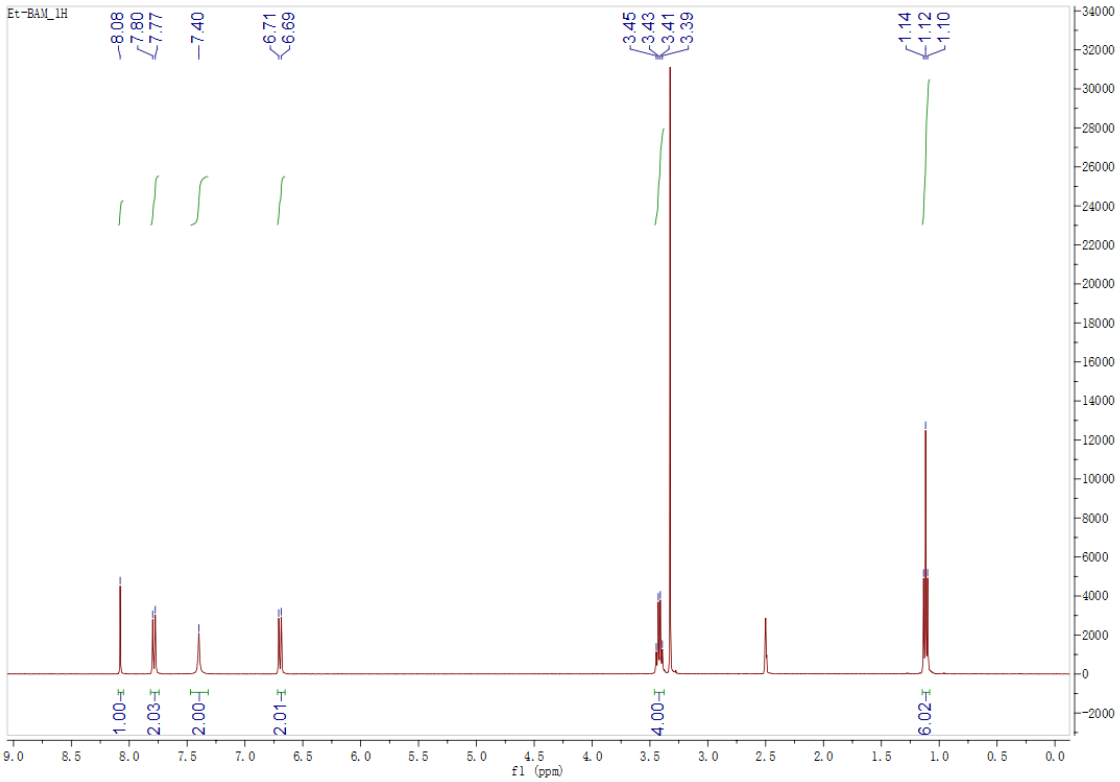


Figure S8. [1]H NMR spectrum of Et-BAM in DMSO-*d6*.

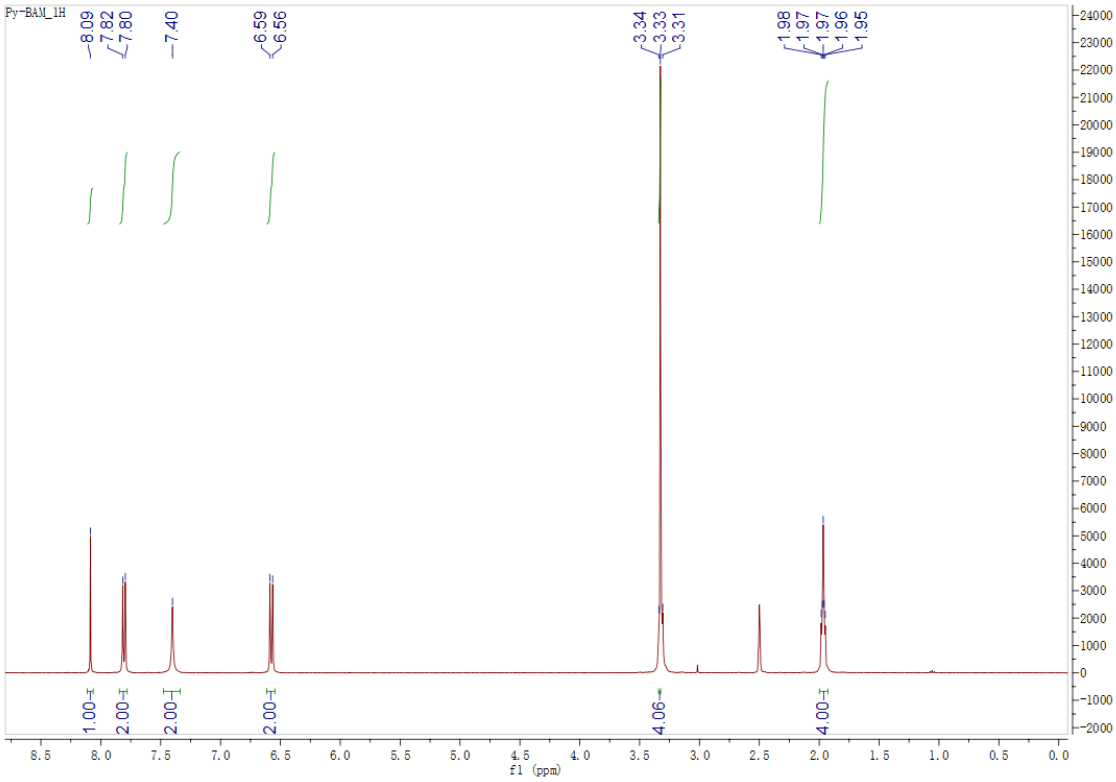


Figure S9. [1]H NMR spectrum of Py-BAM in DMSO-*d6*.

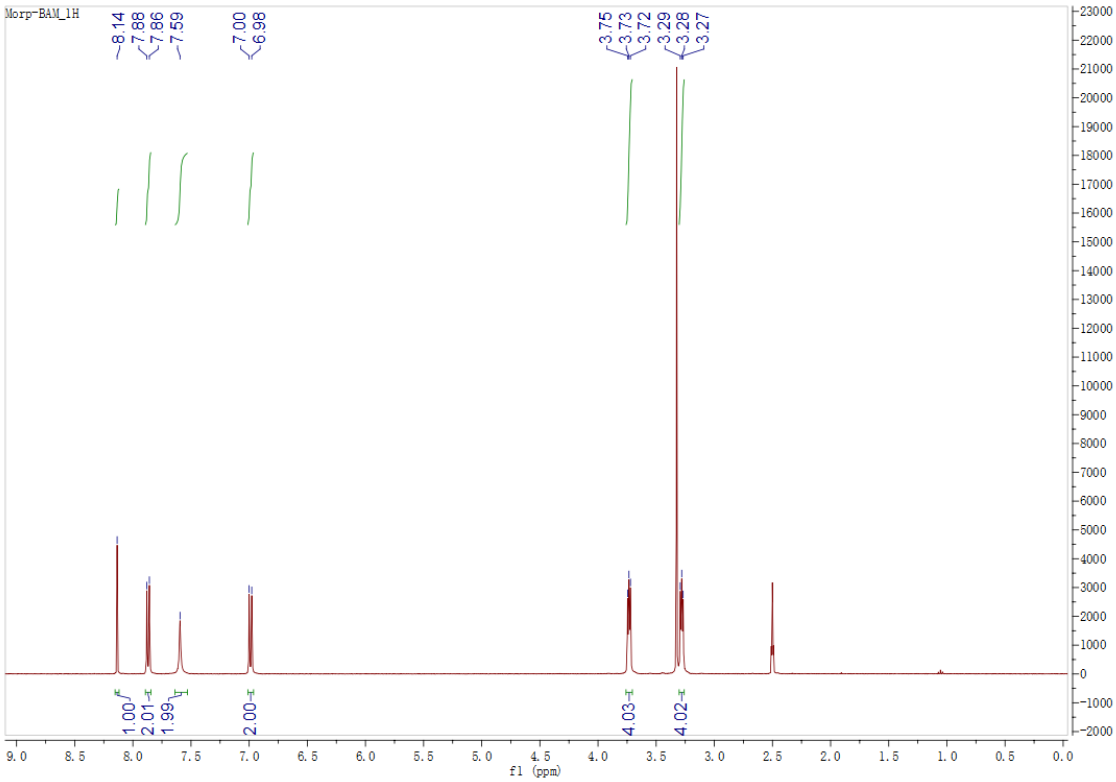


Figure S10. [1]H NMR spectrum of Morp-BAM in DMSO-*d6*.

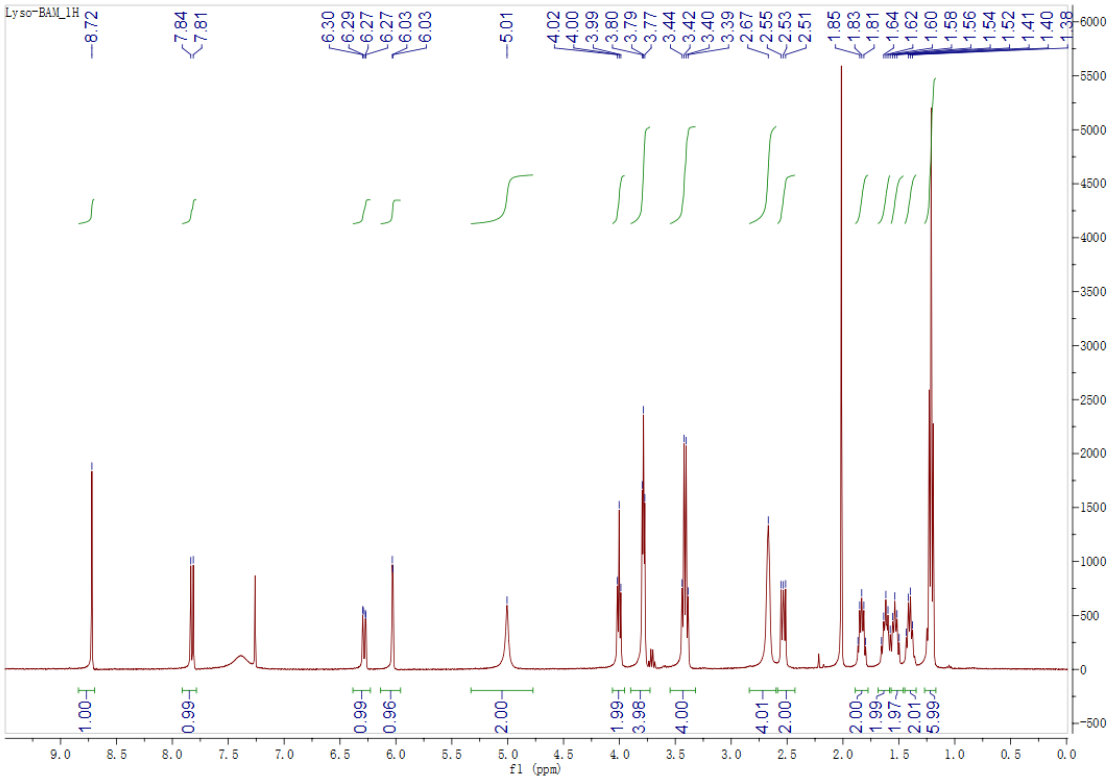


Figure S11. ^{1}H NMR spectrum of Lyso-BAM in $CDCl_3$.

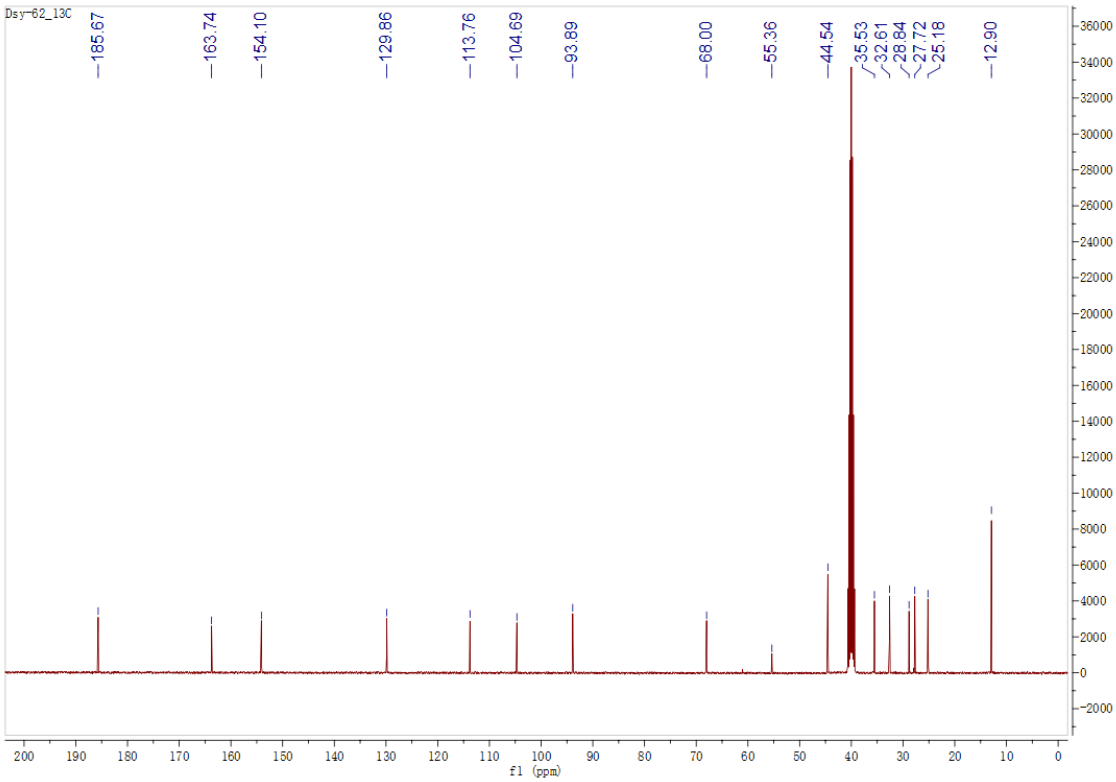


Figure S12. ^{13}C NMR spectrum of compound **2** in DMSO-*d6*.

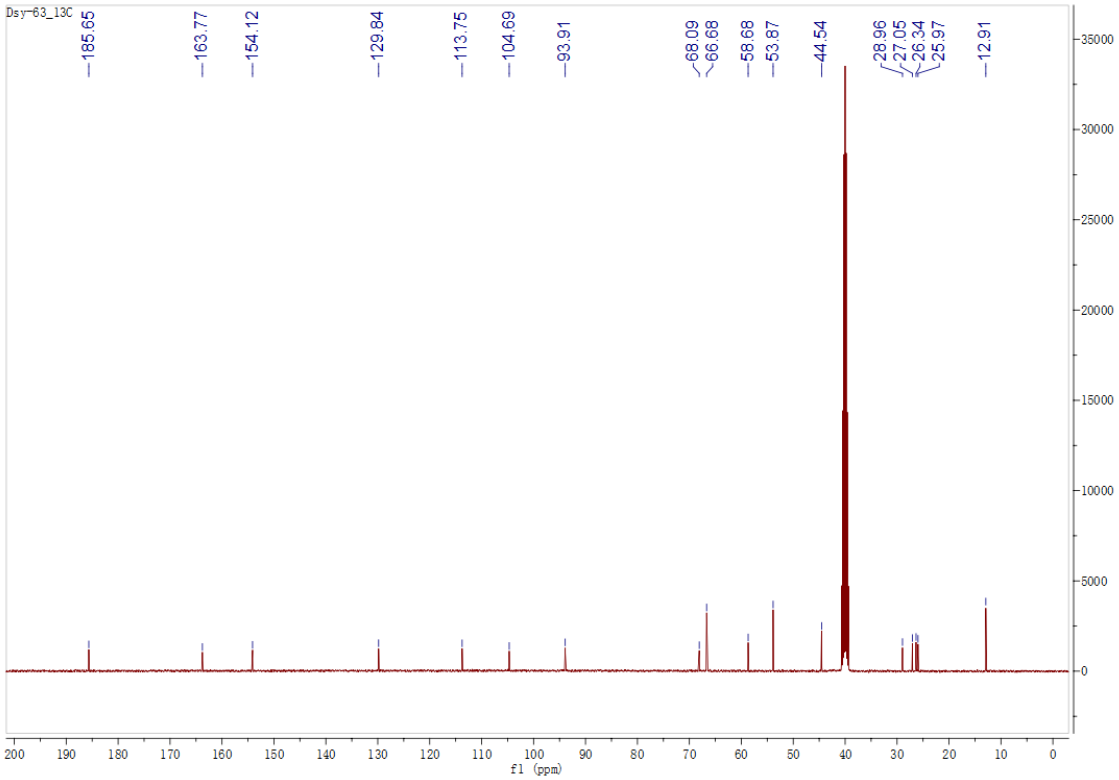


Figure S13. ^{13}C NMR spectrum of compound **3** in DMSO-*d6*.

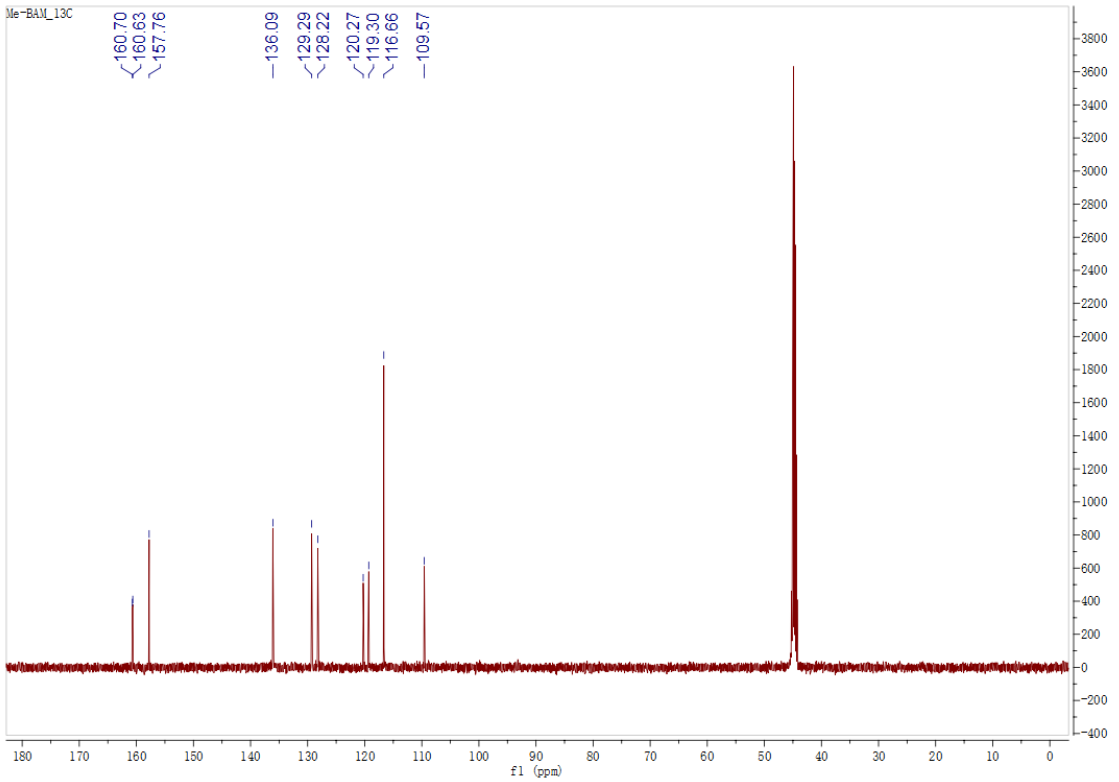


Figure S14. ^{13}C NMR spectrum of Me-BAM in $CDCl_3$.

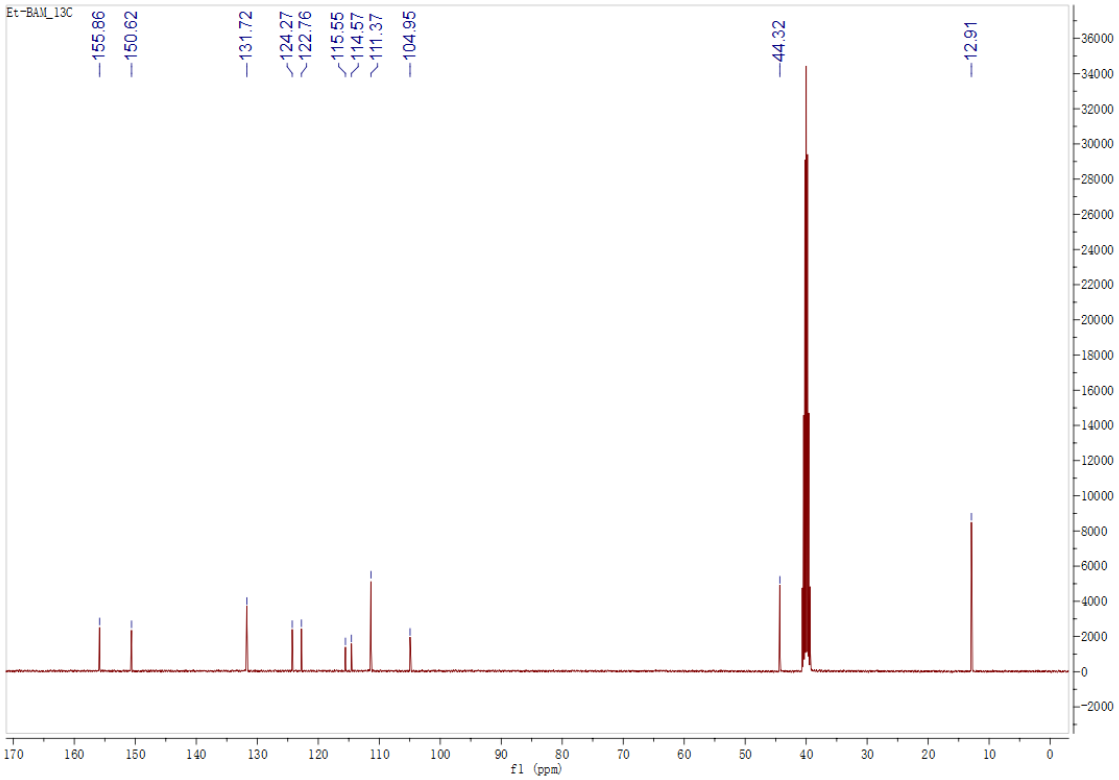


Figure S15. ^{13}C NMR spectrum of Et-BAM in DMSO-*d6*.

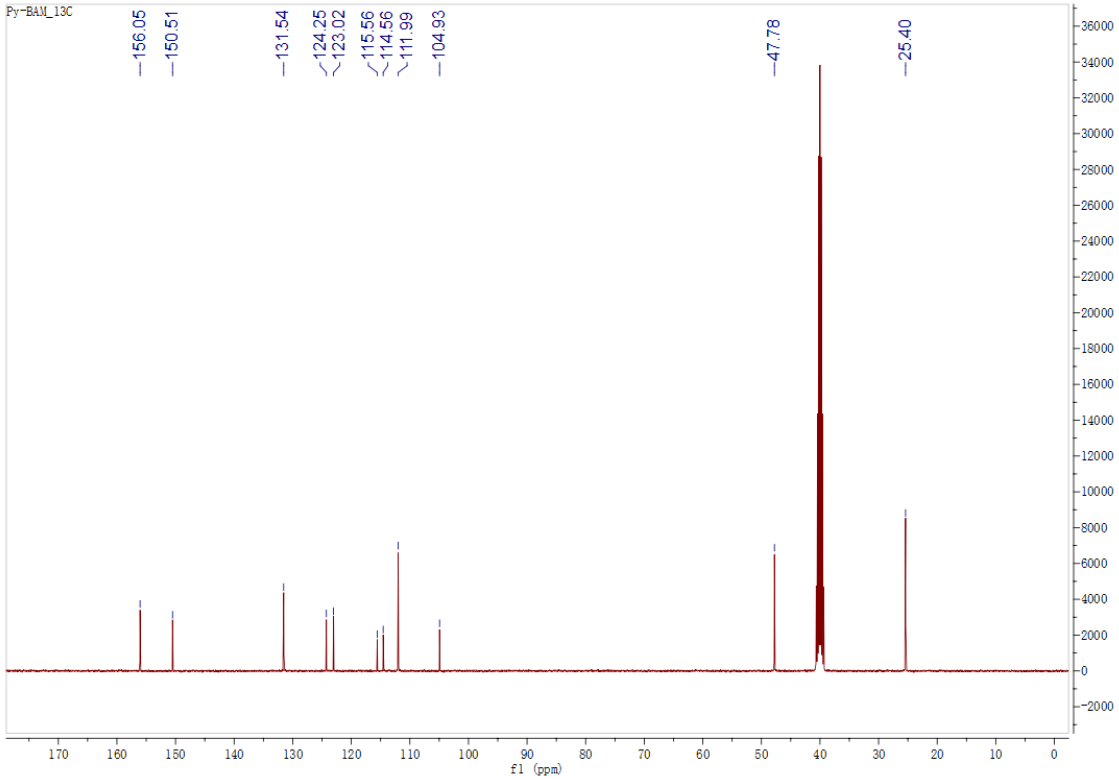


Figure S16. ^{13}C NMR spectrum of Py-BAM in DMSO-*d6*.

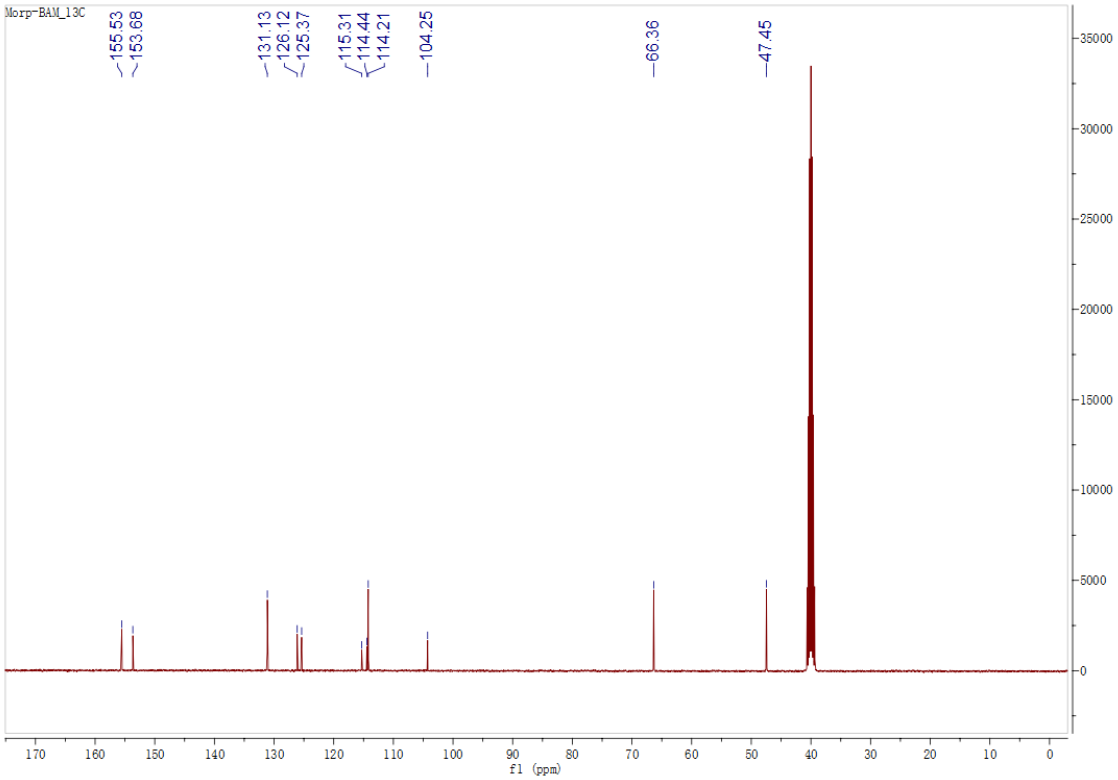


Figure S17. ^{13}C NMR spectrum of Morp-BAM in DMSO-*d6*.

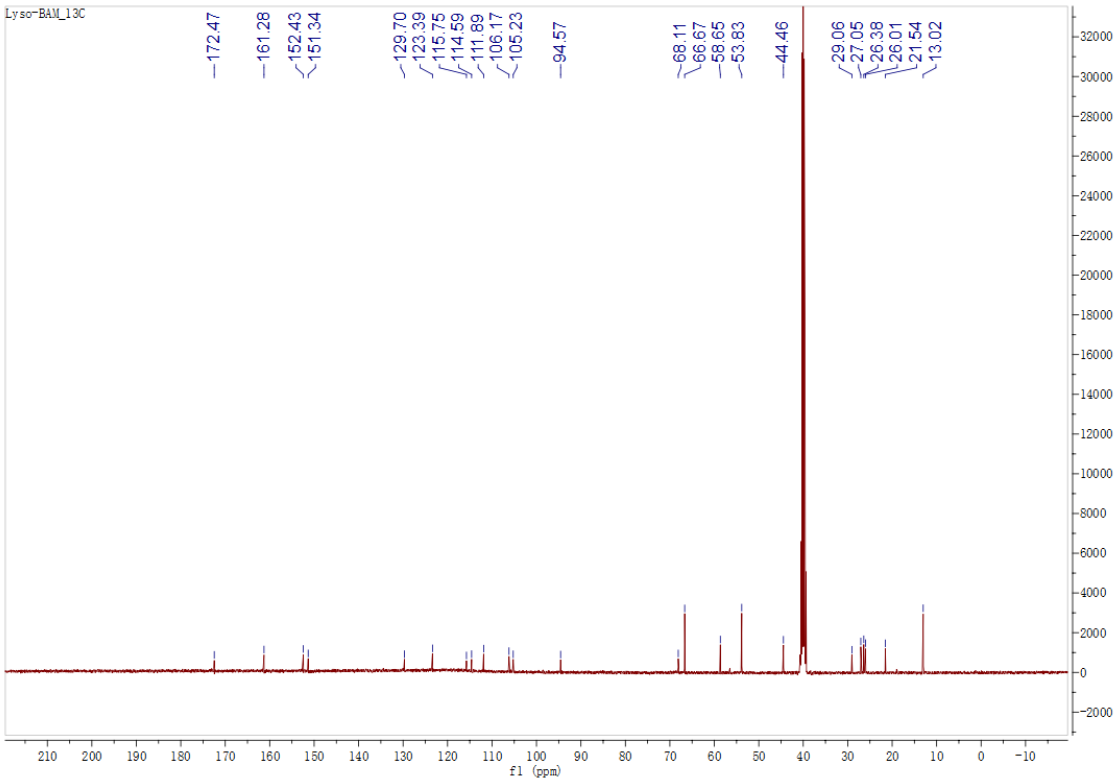


Figure S18. ^{13}C NMR spectrum of Lyso-BAM in DMSO-*d6*.

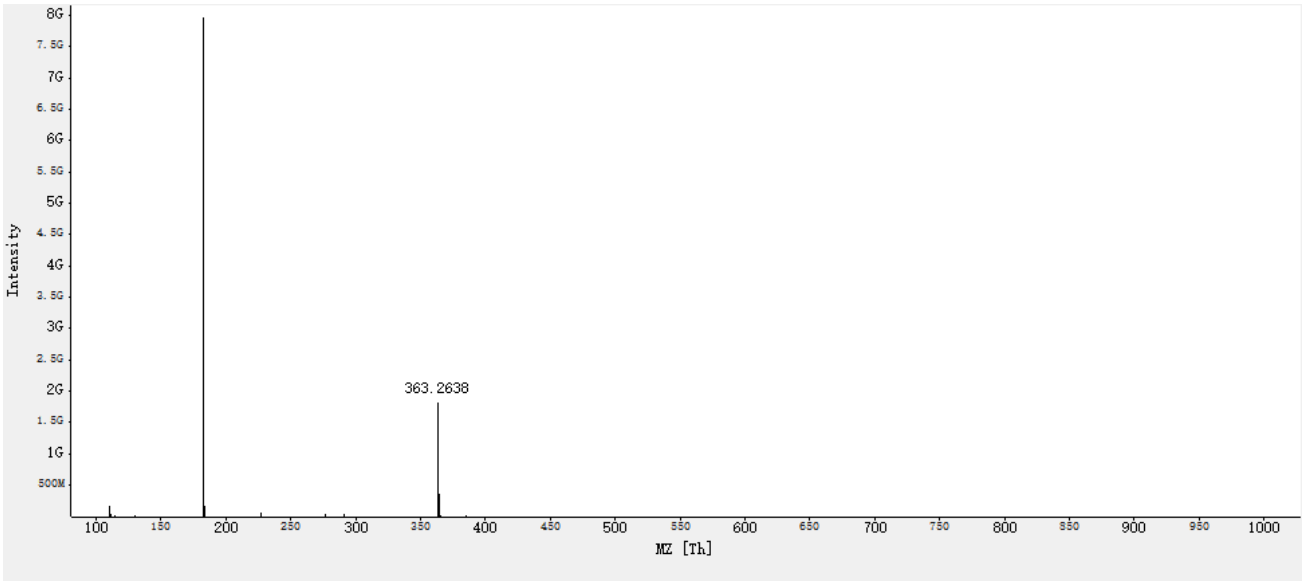


Figure S19. HRMS spectrum of compound **3**.

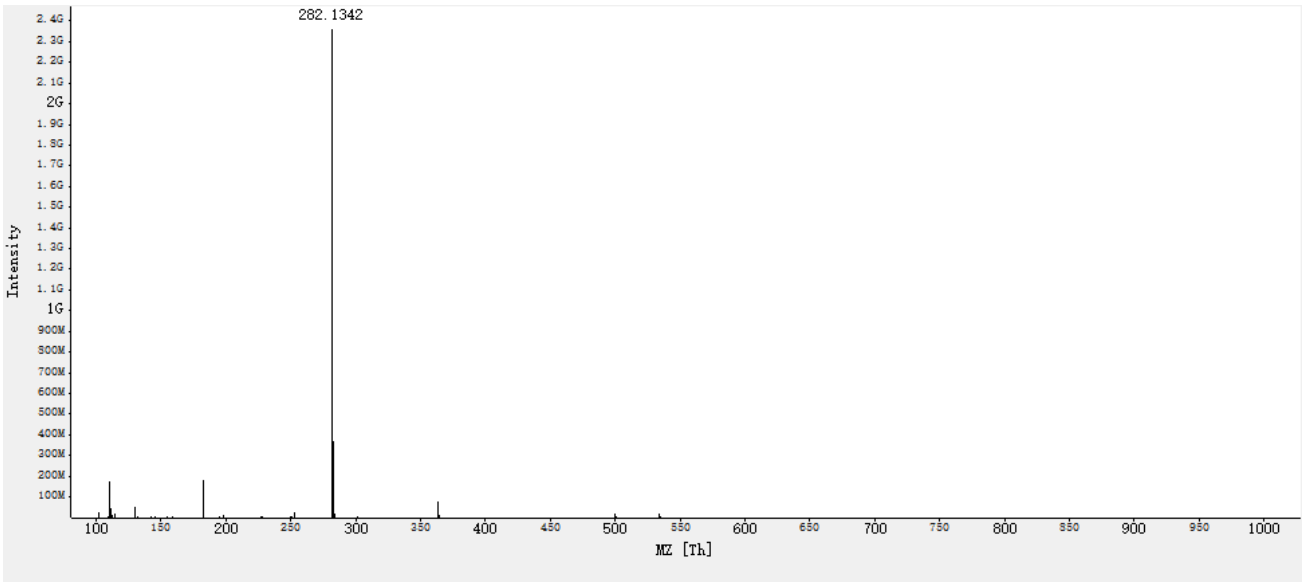


Figure S20. HRMS spectrum of Py-BAM.

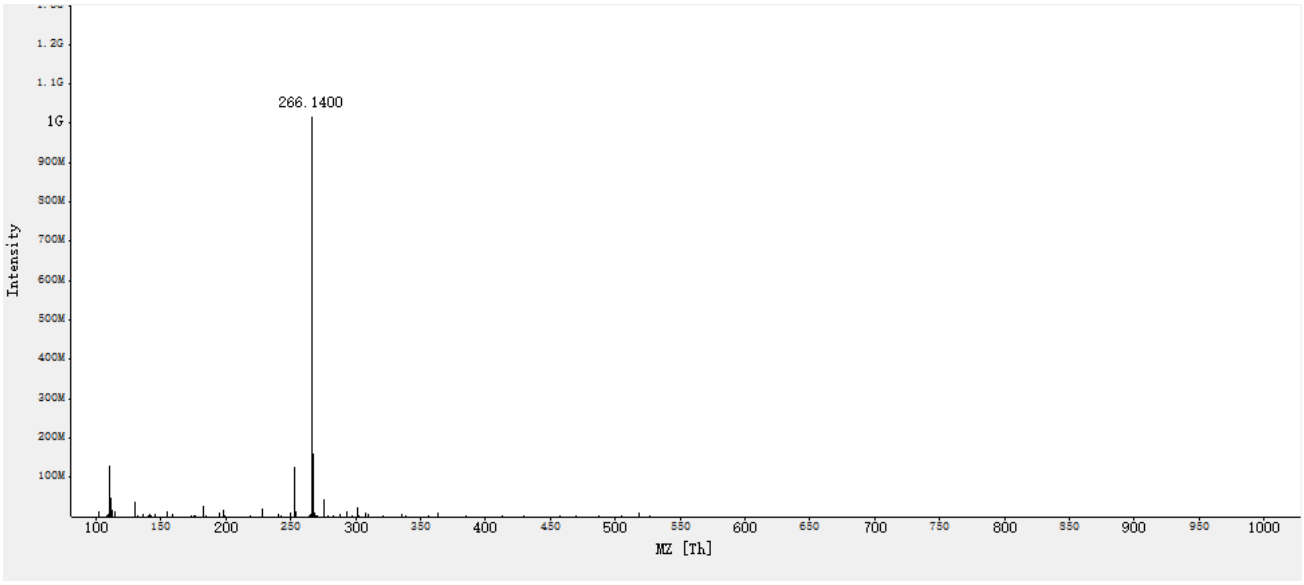


Figure S21. HRMS spectrum of Morp-BAM.

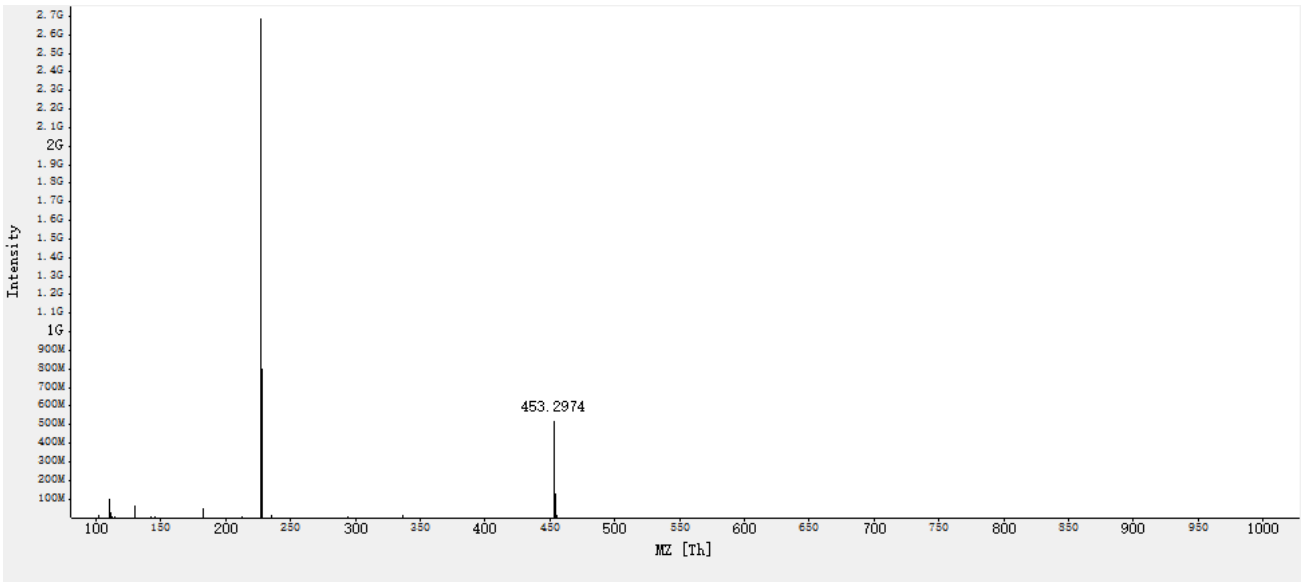


Figure S22. HRMS spectrum of Lyso-BAM.

Reference

1. Shi, B.; Jie, K.; Zhou, Y.; Zhou, J.; Xia, D.; Huang, F., Nanoparticles with Near-Infrared Emission Enhanced by Pillararene-Based Molecular Recognition in Water. *J Am Chem Soc* **2016,** *138* (1), 80-3.
2. Leung, C. W.; Wang, Z.; Zhao, E.; Hong, Y.; Chen, S.; Kwok, R. T.; Leung, A. C.; Wen, R.; Li, B.; Lam, J. W.; Tang, B. Z., A Lysosome-Targeting AIEgen for Autophagy Visualization. *Adv Healthc Mater* **2016,** *5* (4), 427-31.
3. Han, T.; Hong, Y.; Xie, N.; Chen, S.; Zhao, N.; Zhao, E.; Lam, J. W. Y.; Sung, H. H. Y.; Dong, Y.; Tong, B.; Tang, B. Z., Defect-sensitive crystals based on diaminomaleonitrile-functionalized Schiff base with aggregation-enhanced emission. *J Mater Chem C* **2013,** *1* (44).