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A hypoxia-specific and mitochondria-targeted anticancer theranostic agent with high selectivity for cancer cells

Mingxing Hu,‡[a] Chao Yang,‡[a] Yi Luo,[a] Chen Fan,[a] Fangfang Yang,[a] Shuping Yang,[a] Hao Chen,[a] Zhiqiang Cheng,[c] Kun Li, [a, b] and Yongmei Xie*[a]

[a]*State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University, and Collaborative Innovation Center for Biotherapy, Chengdu, Sichuan Province 610041, P. R. China. E-mail: xieym@scu.edu.cn.*
[b]*Key Laboratory of Green Chemistry and Technology, Ministry of Education, College of Chemistry Sichuan University, 29, Wangjiang Road, Chengdu, Sichuan Province 610041, P. R. China.*
[c]*Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, 21205, USA.*

1. Experimental Section

1.1 General remarks for experimental

All materials were obtained from commercial suppliers and used without further purification. All solvents were dried according to the standard methods prior to use. All solvents were either HPLC or spectroscopic grade in the optical spectroscopic studies. ^{1}HNMR, ^{13}CNMR spectra were measured on a Bruker AV-400 (400 MHz) NMR spectrometer. HRMS spectral data were recorded on a Bruker Daltonics Bio TOF mass spectrometer. Fluorescence spectra were obtained using F7000 (HITACHI). HPLC analysis were measured on Waters 2695e equipped with 2998PDA detector.

1.2 Fluorescence analysis

A stock solution of **HMX-1** (10 mM) was prepared in DMSO. All UV/Vis and fluorescence spectra experiments were performed using 10 μM **HMX-1** in PBS buffer solution (pH 7.4, 10 mM) at 298 K.

1.3 Cell lines

All cell lines used in the study were obtained from the American Type Culture Collection. Cells were propagated in RPIM 1640 or DMEM medium containing 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin) in 5% CO_2 at 37℃

1.4 Activation of HMX-1 in cellular under hypoxia

NCM-460 and A375 cells were seeded in confocal dish at the density of 10^5 cells per dish and maintained in the presence of 10% FBS under hypoxia (3% O_2) or normoxia (21% O_2) for 24 h, **HMX-1** (10 μM) was added respectively. After 12h incubation, cells were washed three times with PBS (10 mM). The fluorescence intensities were measured with a confocal microscope.

1.5 Co-localization experiment

A375 cells were incubated with 10 μM of **HMX-1** for 12 h, and then treated with 2 μM of Mito-tracker red for an additional 20 min. After cells were washed three times with PBS (10 mM), cell images were obtained with a confocal microscope.

1.5 Cell toxicity assay

The cell viability of **HMX-1** treated cells was assessed by MTT assay. Briefly, the exponentially growing cells (2-6×10^3 cells/well) were plated in 96-well plates (100 μL/well) and incubated for 24 h. Then the cells were treated with gradient concentrations of **HMX-1** (0, 2.5, 5, 10, 20, 40, and 80 μM). After treatment for 24 h, 20 μL of 5 mg/ml MTT was added to each well, and the plates were incubated at 37 ℃ for additional 2-4 h. The medium was removed and the purple colored precipitates of formazan were dissolved in 150 μL of DMSO. The color absorbance was recorded at 492 nm using a Spectra MAX M5 microplate spectrophotometer (Molecular Devices, CA, U.S.A.). Data shown represents the average of three independent experiments.

1.6 Western blot analysis

A375 cells were treated with **HMX-1** in designed concentration for 24 h, then cells were washed twice with cold PBS and lysed in RIPA buffer. The protein concentrations were measured using the Coomassie brilliant blue G-250 method and equalized before loading. Equal amounts of protein from each sample was subjected to sodium dodecyl sulfate-polyacrylamidegel electrophoresis (SDS-PAGE) gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Bioscience, Piscataway, NJ). Then, the membranes were blocked for 2

h at 37 ℃ and incubated with Bcl-2，Bax and Cleaved caspase-3 antibodies (Amersham, Piscataway, NJ) overnight at 4 ℃. After incubation with the relevant secondary antibodies, the reactive bands were identified using an enhanced chemiluminescence kit (Amersham, Piscataway, NJ).

1.7 Mice and tumor model

All animal experiments have been approved by the Institutional Animal Care and Treatment Committee of Sichuan University in China and were carried out in accordance with the approved guidelines. Female BALB/c nude mice (5-6 weeks old) were obtained from Beijing HFK bioscience Co. Ltd, Beijing, China and were maintained in a specific-pathogen-free (SPF) condition facility with an air-conditioned room at 25 ± 2 ℃ with a relative humidity of 40-70%, and a 12 h light/dark cycle. Mice engrafted subcutaneously with cells (1.0×10^7 cells/100 μL/each) were randomly divided into **HMX-1** or control group when tumor volume was around 100 mm^3 and were given intraperitoneal (IP) injections with **HMX-1** 50 mg/kg or vehicle every 3 days. The tumor size and body weight were measured every 3 days. The mice were sacrificed after 7^{th} injection and tumor excised from animals. Tumor volume is calculated as follows: Volume= $0.5\times a\times b^2$, where a (mm) was the length and b (mm) is the width of the tumor.

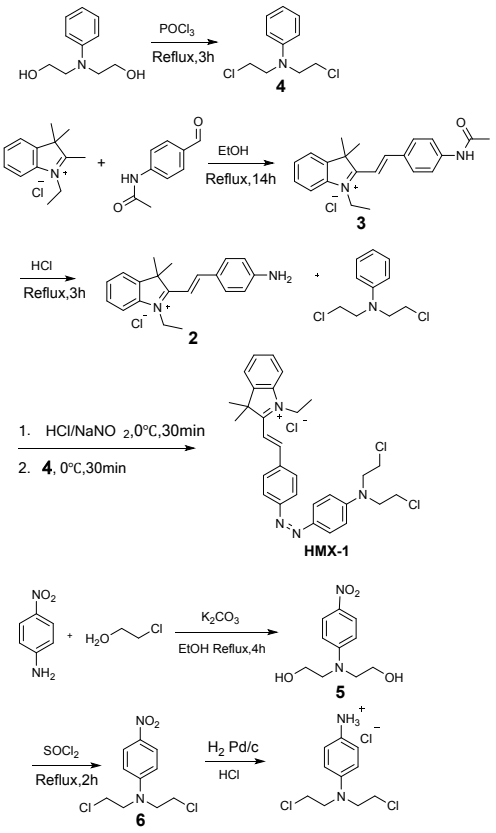
	IC_{50}(μM)				
Cell lines	NCM-460	A375	MDA-MB-231	A549	DU145
Normoxia	53.4 ± 1.5	23.4 ± 1.2	27.8 ± 1.2	31.3 ± 1.2	35.3 ± 1.3
3% Hypoxia	38.5 ± 1.3	11.2 ± 1.1	16.3 ± 1.2	18.3 ± 1.1	19.6 ± 1.1

Table S1 IC_{50} values for **HMX-1** in different cell lines under normoxia and hypoxia (3% O_2). Cytotoxicity determined by MTT assay after 24 h treatment. Data were representative of three independent experiments.

	IC_{50}(μM)	
Cell lines	NCM-460	A375
Normoxia	25.1 ± 1.3	27.2 ± 1.2
3% Hypoxia	20.2 ± 1.2	22.6 ± 1.1

Table S2 IC_{50} values for **aniline nitrogen mustard hydrochloride** in A375 and NCM-460 under normoxia and hypoxia (3% O_2). Cytotoxicity determined by MTT assay after 24 h treatment. Data were representative of three independent experiments

1.8 Synthesis and characterization of HMX-1 and aniline nitrogen mustard hydrochloride (spectra could be found in Fig S3-S19).



Scheme S1. Synthesis route of **HMX-1** and **aniline nitrogen mustard hydrochloride**

(1) Synthesis of compound 4

2,2'-(Phenylimino)diethanol (3.6 g, 20 mmol) was dissolved in 20 mL $POCl_3$ and reflux for 3h. The reaction mixture was concentrated under reduced pressure and diluted with 100 ml of CH_2Cl_2 and then washed with saturated brines (3 × 50 mL), dried over $MgSO_4$ and concentrated under reduced pressure. Purification by column chromatography yielded 2.82 g (yield, 65 %) of the title product. ^{1}H NMR (400 MHz, $CDCl_3$), δ 7.28 - 7.22 (m, 2H), 6.77 (dt, J = 7.3, 1.9 Hz, 1H), 6.68 (d, J = 5.7 Hz, 2H), 3.75 - 3.59 (m, 8H) ppm. HRMS calcd for $C_{10}H_{13}Cl_2N$ $[M + H]^+$: 218.0459, found: 218.0525.

(2) Synthesis of compound 3

To a solution of 2,3,3-trimethyl-1-ethyl-3H-indol-1-ium iodide (329 mg, 1.0 mmol) in anhydrous EtOH (10 mL) was added p-acetamidobenzaldehyde (163 mg, 1.0 mmol) and reflux for 14h. The mixture was filtrated after cooled in refrigerator, the solid filter washed with a minimal amount of cold EtOH, dried in air, affording the desired purple red product 270 mg (yield, 73%). ^{1}H NMR (400 MHz, DMSO-d_6), δ 10.44 (s, 1H), 8.43 (d, J = 6.2 Hz, 1H), 8.25 - 8.20 (m, 2H), 7.91 (dd, J = 5.4, 2.2 Hz, 2H), 7.81 (d, J = 6.6 Hz, 2H), 7.65 -7.60 (m, 2H), 4.70 (d, J = 6.8 Hz, 2H), 2.13 (s, 3H), 1.80 (s, 6H), 1.46 (t, J = 5.2 Hz, 3H) ppm. HRMS calcd for $C_{22}H_{25}N_2O$ $[M]^+$: 333.1961, found: 333.1866.

(3) Synthesis of compound 2

3 (368 mg, 1 mmol) was dissolved in hydrochloric acid (37%, 10 mL) and reflux for 3h. After cooling to room temperature, the solvent was removed by evaporation. The crude product was pure

enough without purification. 312 mg Brick-red product was obtained (yield, 95%). ^{1}H NMR (400 MHz, DMSO-d_6), δ 8.28 (d, J = 5.5 Hz, 1H), 8.02 (s, 2H), 7.78 (d, J = 7.3 Hz, 1H), 7.72 (d, J = 8.0 Hz, 1H), 7.55 (t, J = 7.7 Hz, 1H), 7.47 (t, J = 7.4 Hz, 1H), 7.23 - 7.05 (m, 3H), 6.73 (d, J = 8.0 Hz, 2H), 4.53 (d, J = 6.2 Hz, 2H), 1.74 (s, 6H), 1.38 (t, J = 7.1 Hz, 3H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6), δ 179.34, 156.81, 155.48, 143.19, 141.21, 129.29, 127.89, 123.33, 122.89, 114.56, 113.81, 104.20, 56.49, 51.30, 26.94, 19.03, 13.63 ppm. HRMS calcd for $C_{20}H_{23}N_2$ $[M]^+$: 291.1856, found: 291.1858.

(4) Synthesis of HMX-1

To a stirred solution of 2 (326 mg, 1 mmol) in 20 mL (ACN/ /H_2O = 1/1) at 0°C under N_2 atmosphere, 75 mg (1.05 mmol) $NaNO_2$ was added and the solution was stirred for 10 min after which 115 mg (1 mmol) TFA was added and the solution was stirred for another 30 min. Subsequently 435 mg of 4 (2 mmol) in 5 mL ACN was added and the mixture was stirred for 4 h at 0 °C, after which 100 mL water was added. The mixture was extracted with DCM (3 × 50 mL), the organic layers were combined, dried over with $MgSO_4$ and the solvent was removed in vacuo. **HMX-1** was isolated after chromatographic separation (silica gel, Methanol/DCM = 1:50) in a 54% yield (300 mg). ^{1}H NMR (400 MHz, DMSO-d_6), δ 8.53 (d, J = 7.3 Hz, 1H), 8.42 (d, J = 8.5 Hz, 2H), 7.99 - 7.85 (m, 6H), 7.79 (d, J = 8.3 Hz, 1H), 7.67 - 7.64 (m, 2H), 7.00 (d, J = 7.1 Hz, 2H), 4.77 (d, J = 7.1 Hz, 2H), 3.87 (dt, J = 7.2, 3.6 Hz, 8H), 1.83 (s, 6H), 1.49 (t, J = 7.2 Hz, 3H) ppm. ^{13}C NMR (100 MHz, CD_3OD), δ 153.68, 150.39, 143.91, 135.06, 131.41, 129.79, 129.26, 125.62, 122.78, 114.68, 111.75, 52.78, 52.61, 42.33, 40.17, 25.10, 12.76 ppm. HRMS calcd for $C_{30}H_{33}Cl_2N_4$ $[M]^+$: 519.2077, found: 519.2082.

(5) Synthesis of compound 5

To a solution of 4-nitroaniline (1.38 mg, 10 mmol) in anhydrous EtOH (50 mL) was added potassium carbonate (4.14 g, 30.0 mmol) and 2-chloroethanol (1.61g, 20mmol), the mixture was refluxed for 4h. The mixture was filtrated after cooled in refrigerator, washed with a minimal amount of cold EtOH, dried, affording the desired yellow solid product 1.95 g (yield, 86%). ^{1}H NMR (400 MHz, DMSO-d_6), δ 8.01 (d, J = 9.4 Hz, 2H), 6.82 (d, J = 9.2 Hz, 2H), 4.84 (d, J = 4.9 Hz, 2H), 3.59 (t, J = 6.1 Hz, 8H) ppm.

(6) Synthesis of compound 6

5 (904 mg, 4mmol) was dissolved in 10 mL $SOCl_2$ and reflux for 3 h. The reaction mixture was concentrated under reduced pressure and diluted with 100 ml of CH_2Cl_2 and then washed with saturated brines (3 × 50 mL), dried over $MgSO_4$ and concentrated under reduced. The product was directly used for next step without purification.

(7) Synthesis of compound aniline nitrogen mustard hydrochloride

A mixture of compound **6** (1048 mg, 4 mmol) and 10% Pd/C (200 mg) in methanol (20 mL) was stirred overnight under H_2 atmosphere. The reaction mixture was filtered over Celite, and the filtrate was concentrated in vacuo. The residual oil was dissolve in saturated hydrochloric acid ethanol solution and stirred for 30 min. Appropriate amount of diethyl ether was added under stir to obtain aniline nitrogen mustard hydrochloride 704 mg (yield, 68%) as white solid. ^{1}H NMR (400 MHz, DMSO-d_6), δ 10.01 (s, 3H), 7.21 (d, J=8.9 Hz, 2H), 6.83 (d, J=8.9 Hz, 2H), 3.73 (s, 8H). ^{13}C NMR (100 MHz, DMSO-d_6), δ 146.62, 124.65, 120.94, 112.91, 52.43, 41.45 ppm. HRMS calcd for $C_{10}H_{15}N_2Cl_2$ $[M]^+$: 233.0607, found: 233.40.

2. Cell viability and others

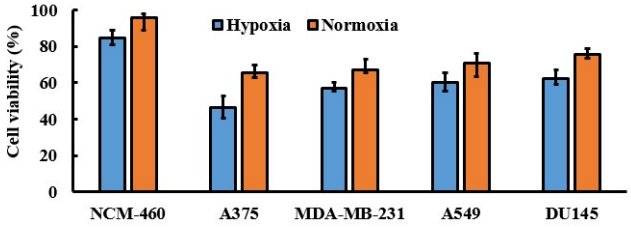


Fig. S1 Comparison of cell viability in hypoxia and normoxia after treatment with **HMX-1** (10 μM). Cells were seeded in 96-well plates and treated with **HMX-1** for 24 h. The MTT assay was applied to assess cell viability. Graphs were representative of 3 independent experiments.

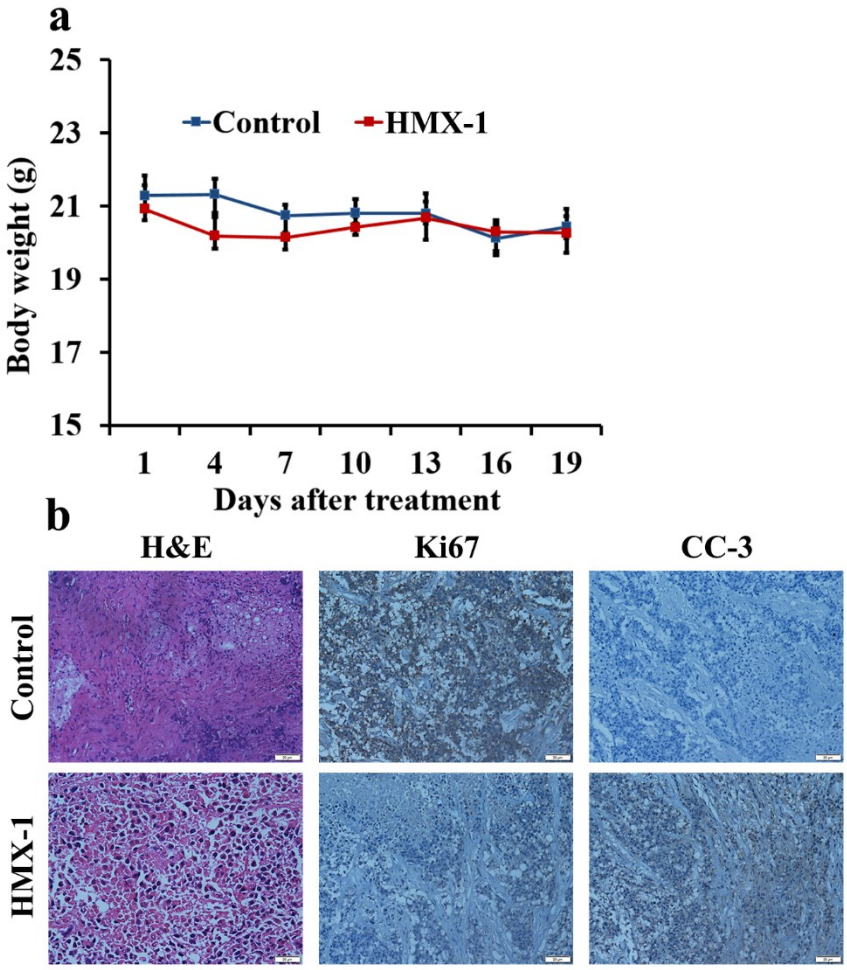


Fig. S2 (a) Body weight of **HMX-1** group and control group after treatment. Data were expressed as means ± SD. (b) Tumor tissues were dissected at day 19 after treatment, H&E, Ki67 and CC-3 staining analysis of tumor tissues were subsequently conducted. Scale bar, 20 μm.

3. Spectra

^{1}H, ^{13}C NMR, ESI-MS and HPLC spectra

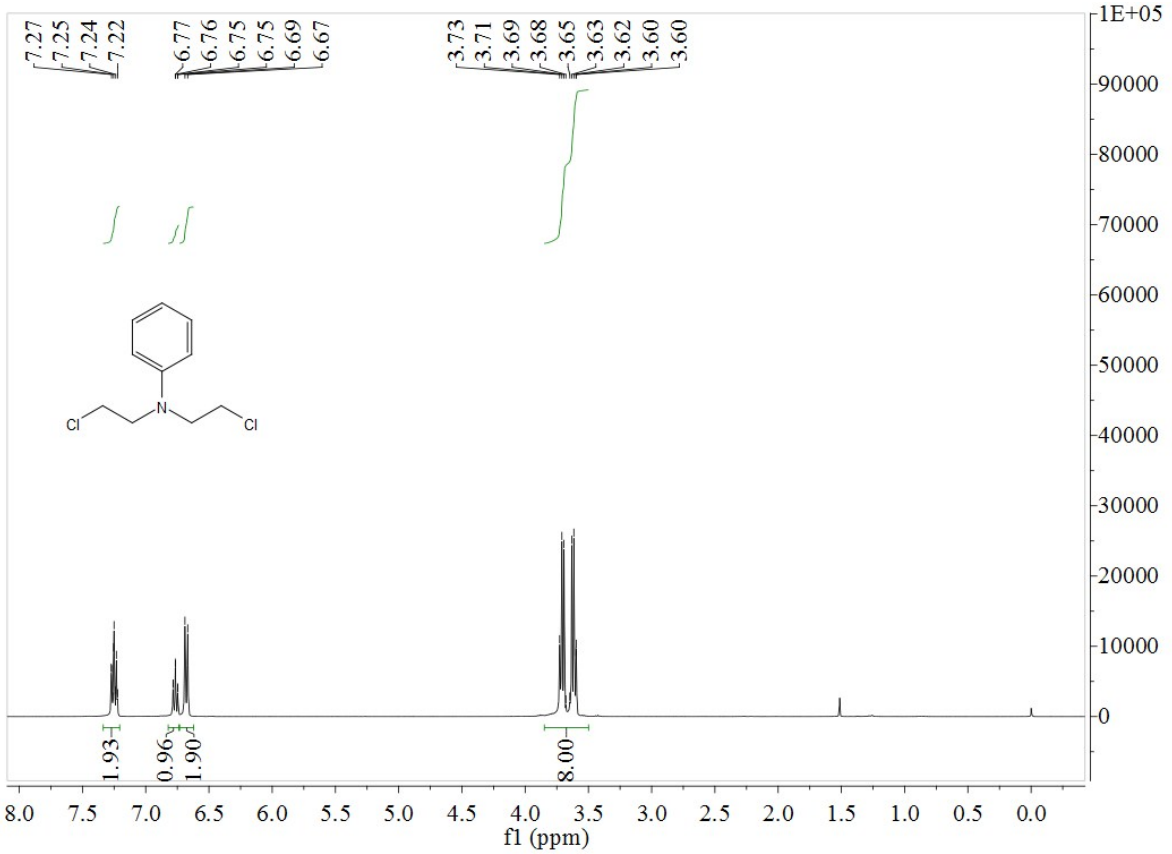


Fig. S3 ^{1}H NMR of **4**.

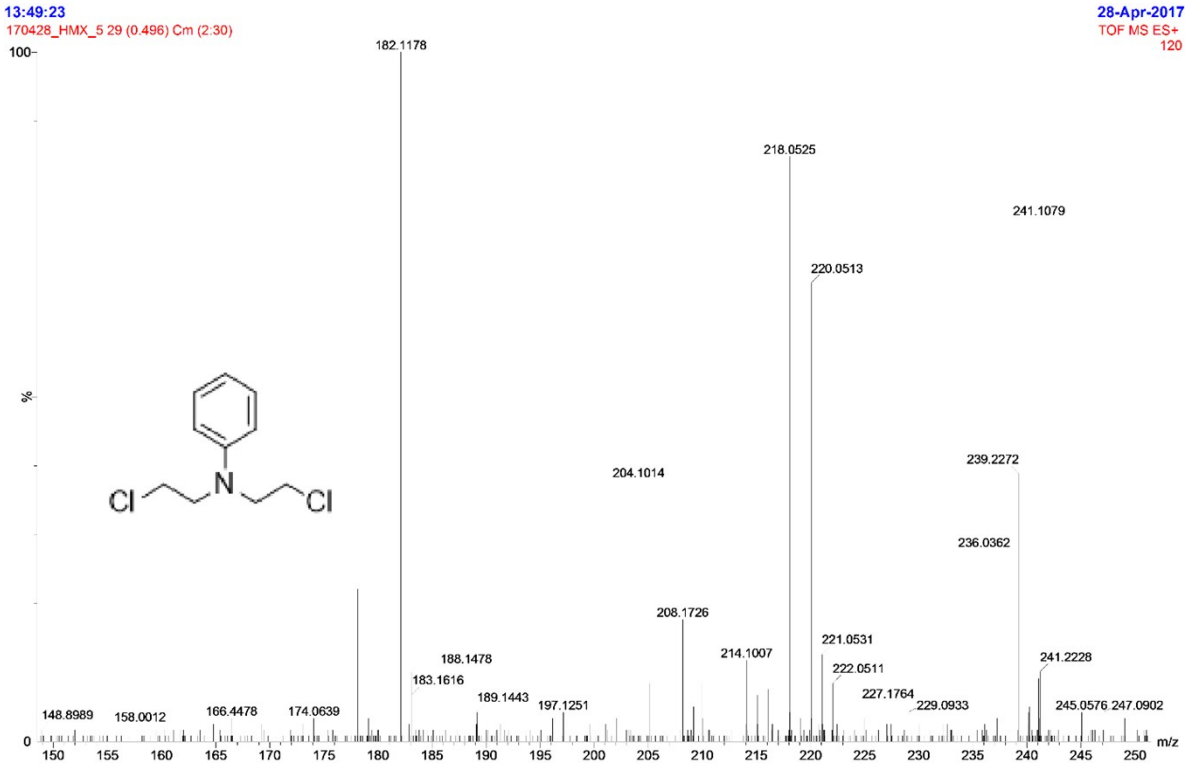


Fig. S4 MS spectrum of **4.**

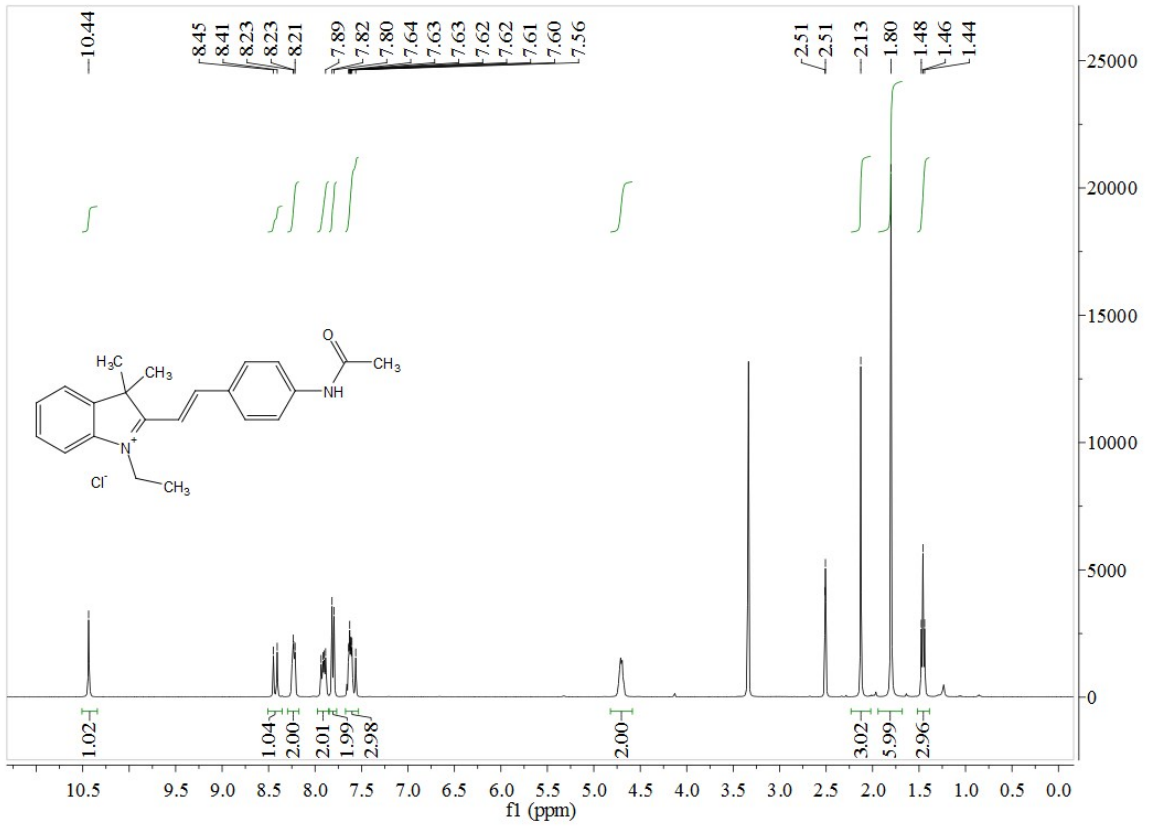


Fig. S5 ^{1}H NMR of **3**.

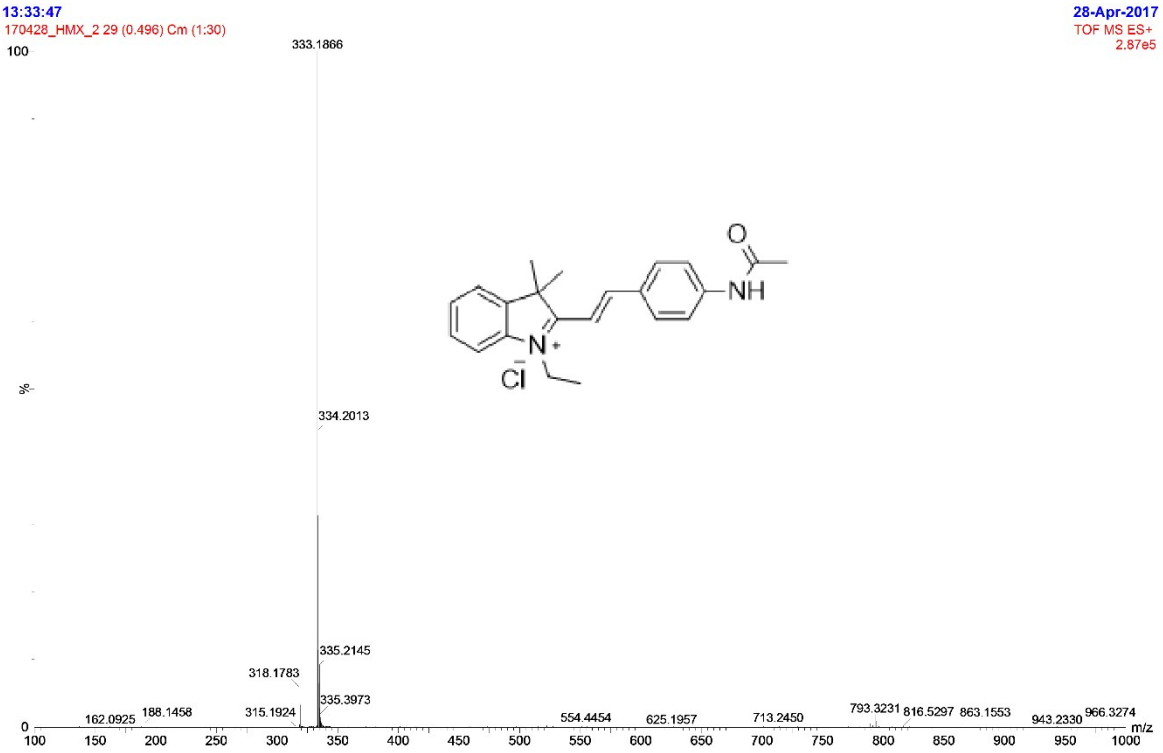


Fig. S6 MS spectrum of **3.**

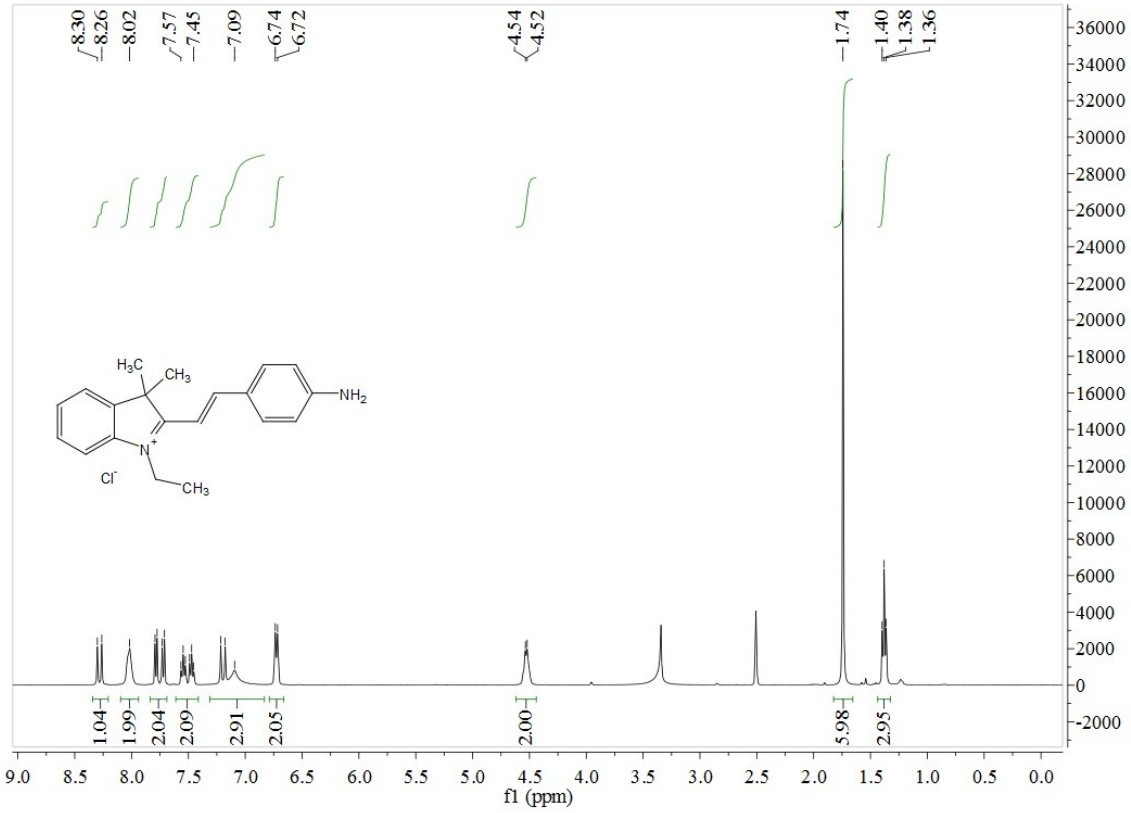


Fig. S7 ^{1}H NMR of **2**.

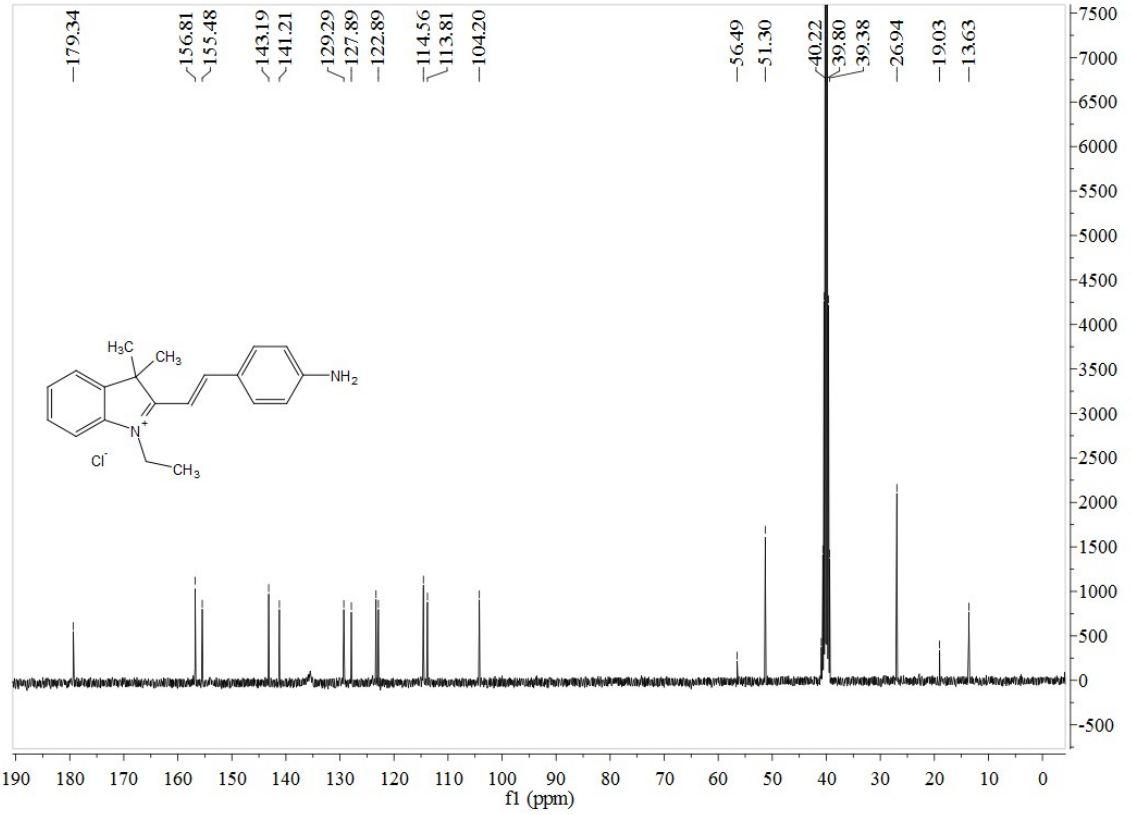


Fig. S8 ^{13}C NMR of **2**.

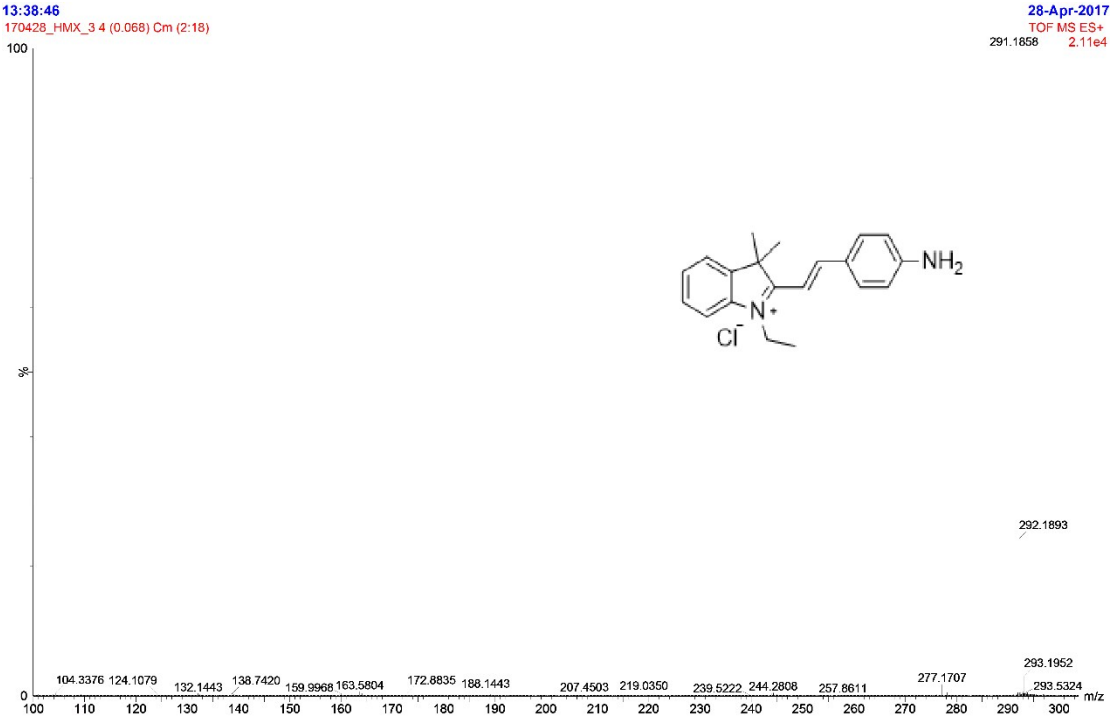


Fig. S9 MS spectrum of **2**.

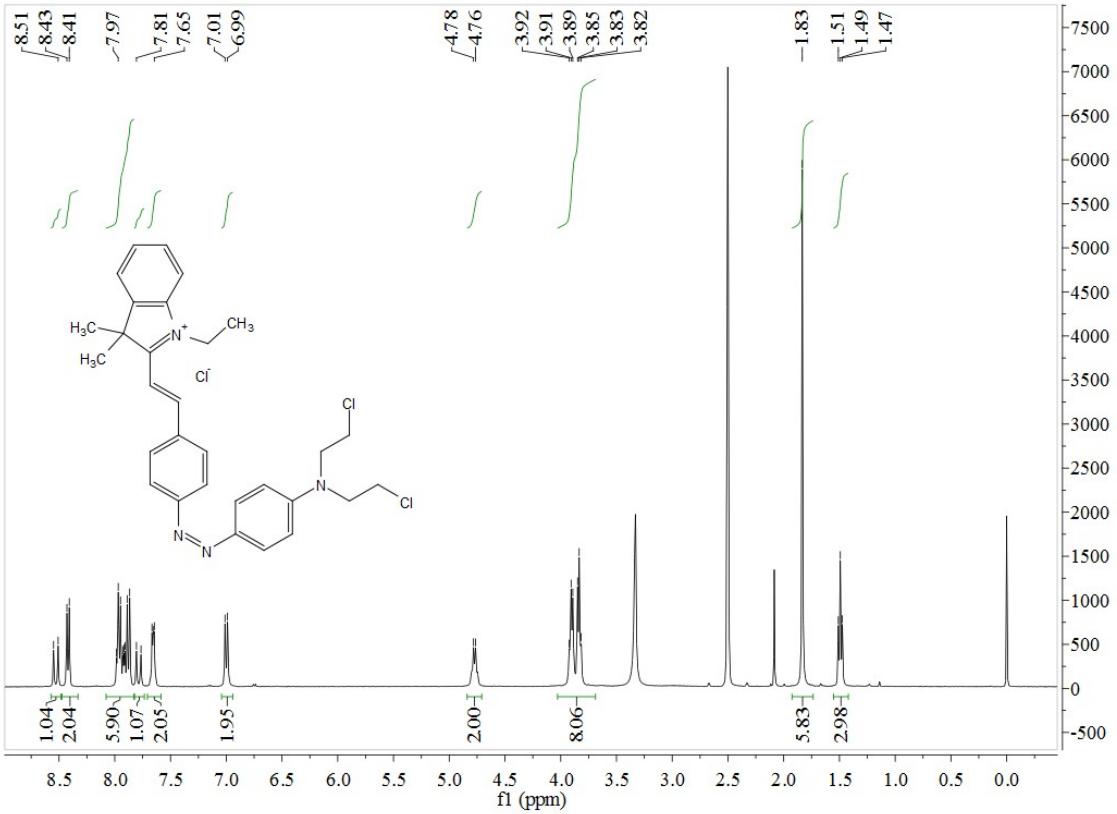


Fig. S10 ^{1}H NMR of **HMX-1**.

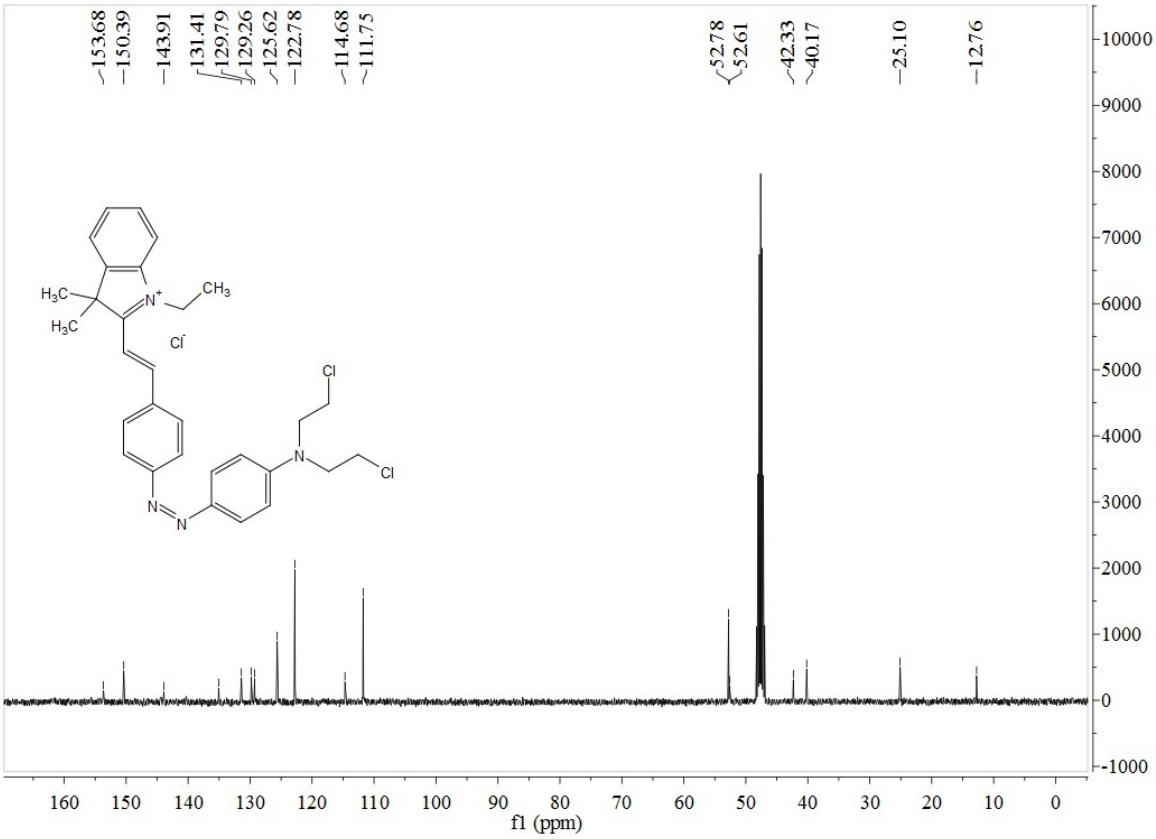


Fig. 11 ^{13}C NMR of **HMX-1**.

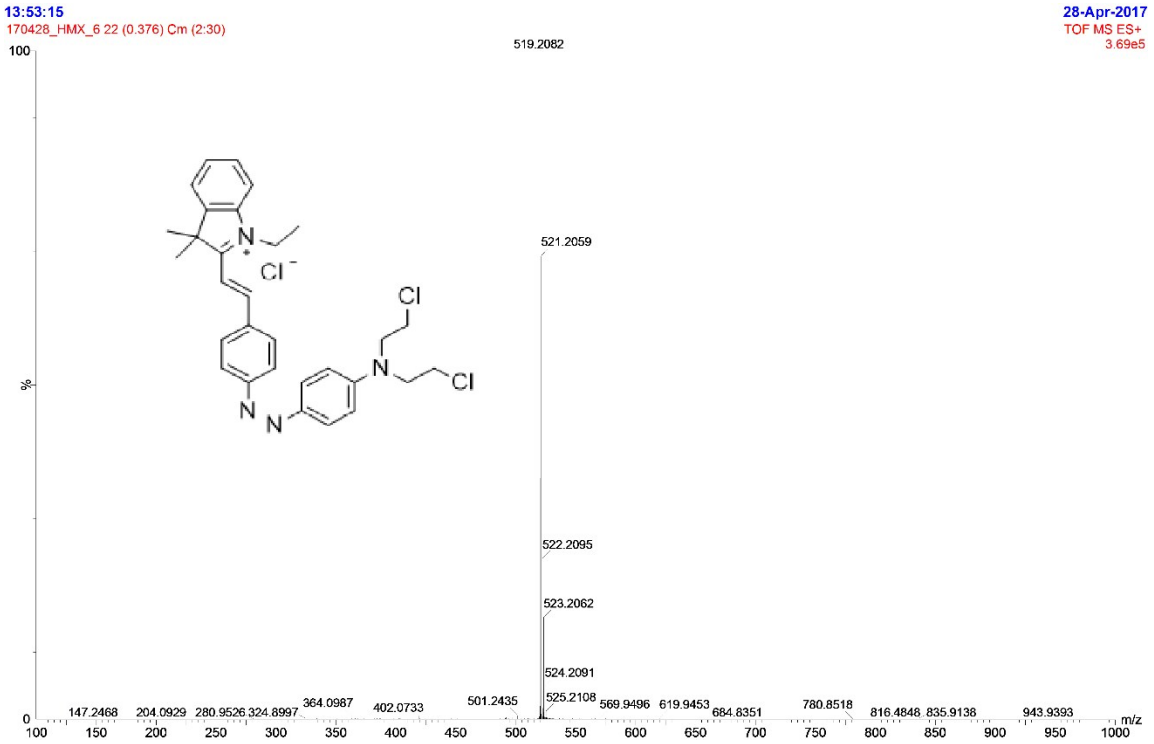


Fig. S12 MS spectrum of **HMX-1**.

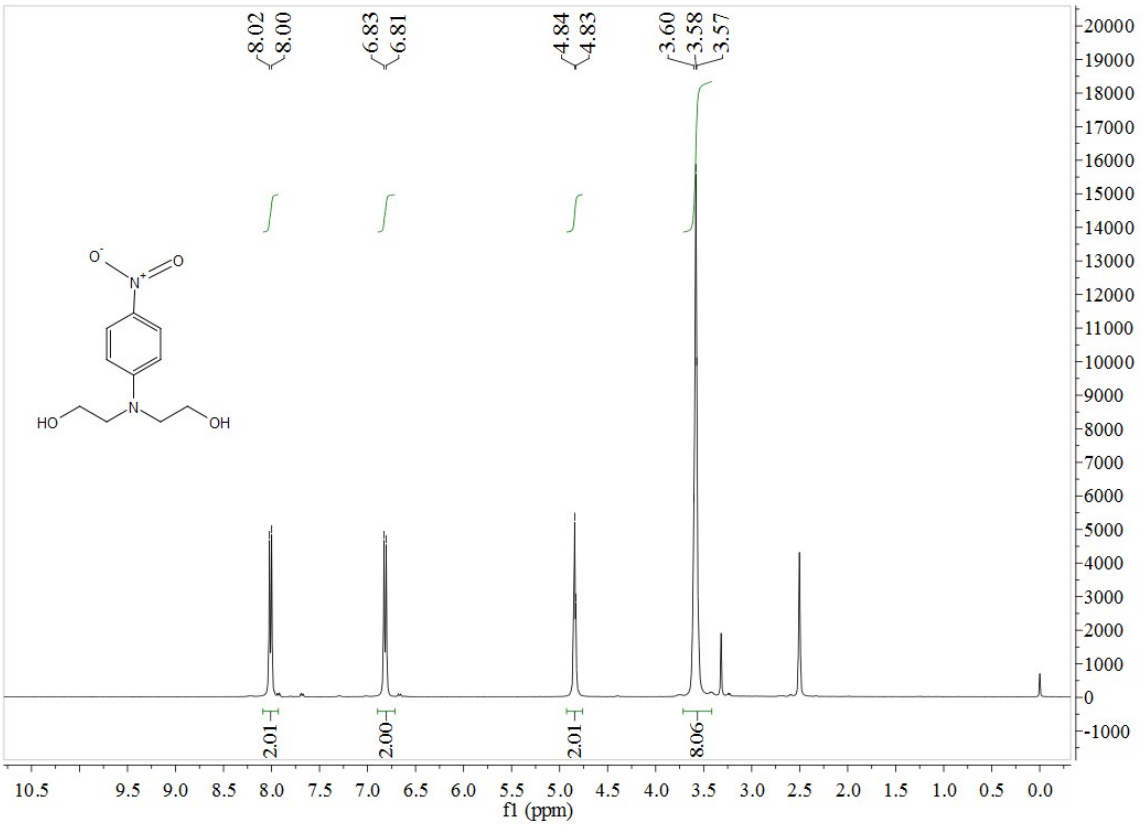


Fig. S13 ^{1}H NMR of **5**.

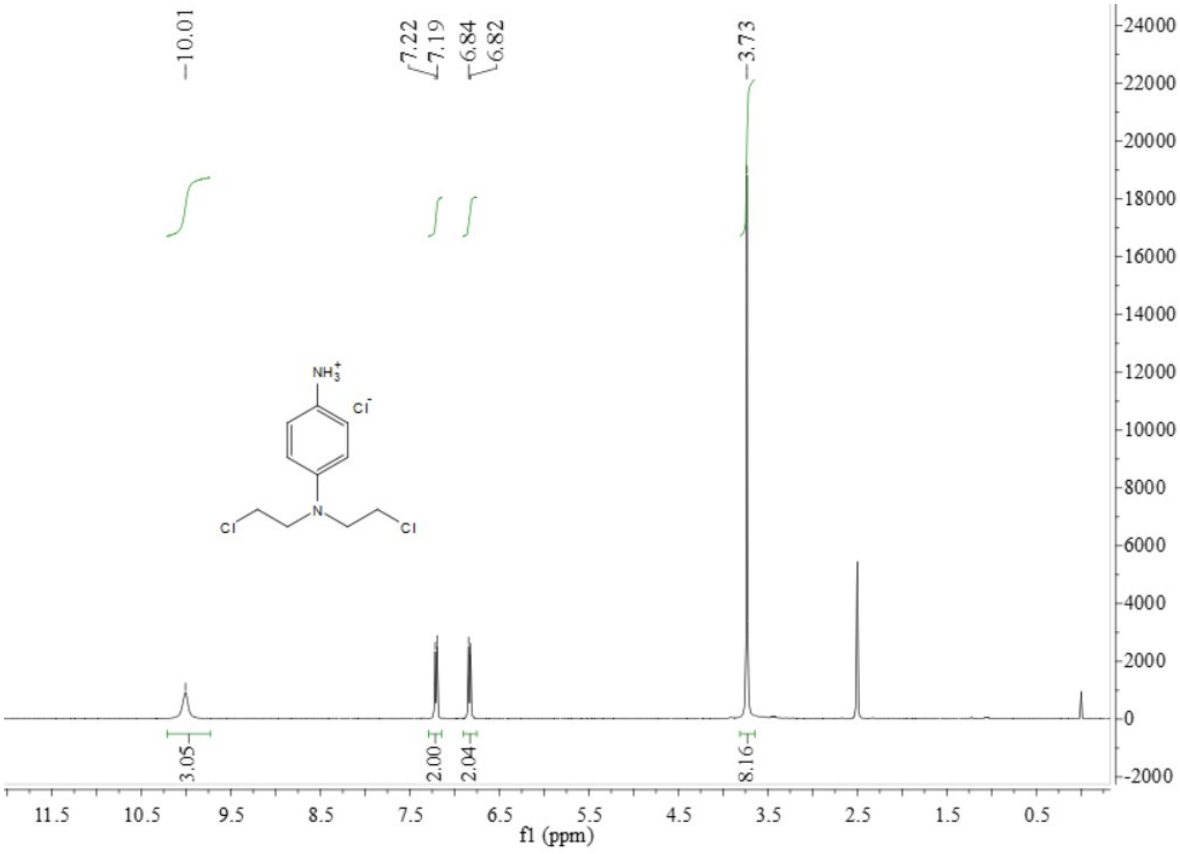


Fig. S14 ^{1}H NMR of **aniline nitrogen mustard hydrochloride**.

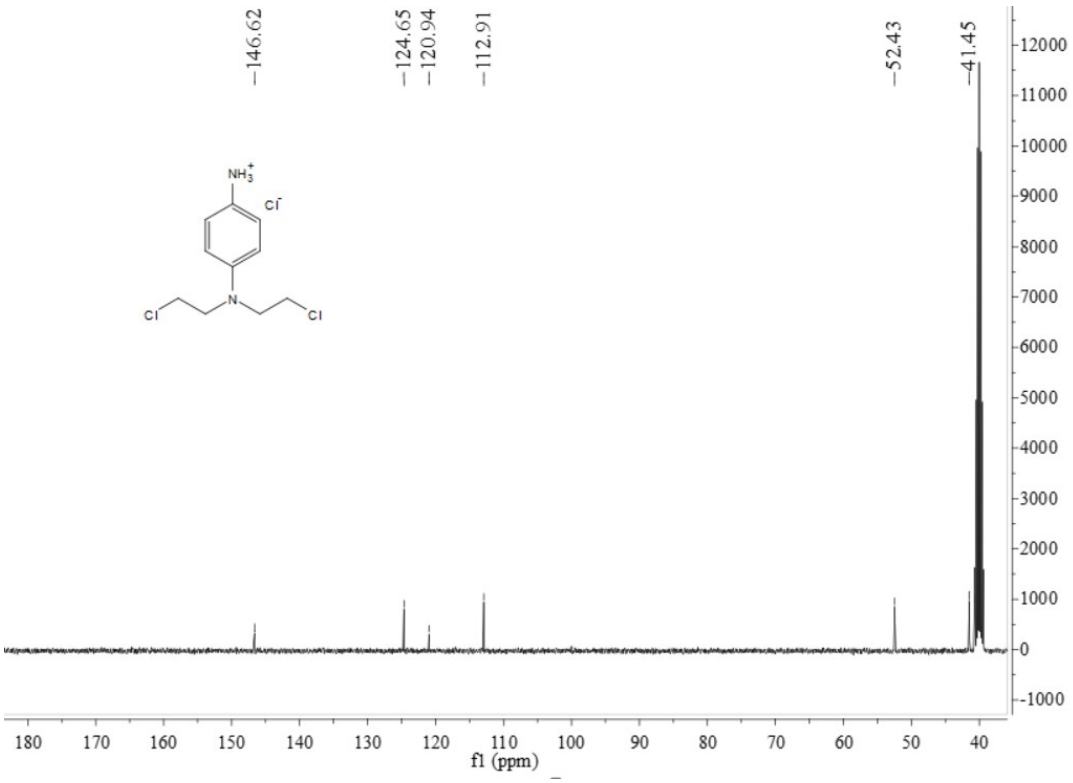


Fig. S15 ^{13}C NMR of **aniline nitrogen mustard hydrochloride**.

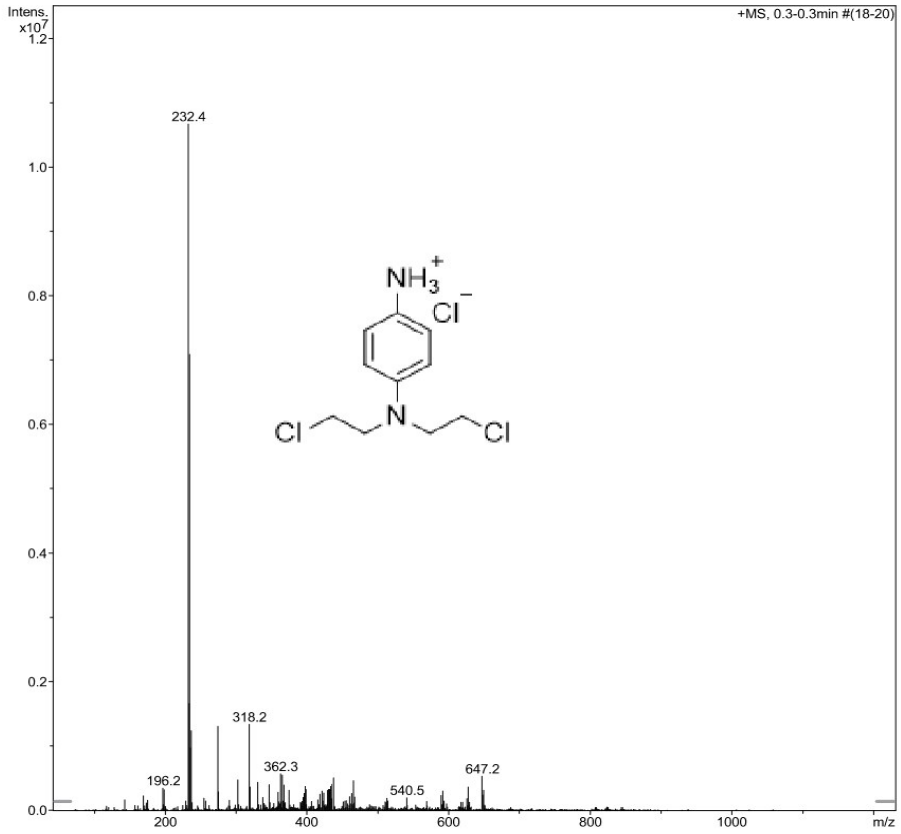


Fig. S16 MS spectrum of **aniline nitrogen mustard hydrochloride**.

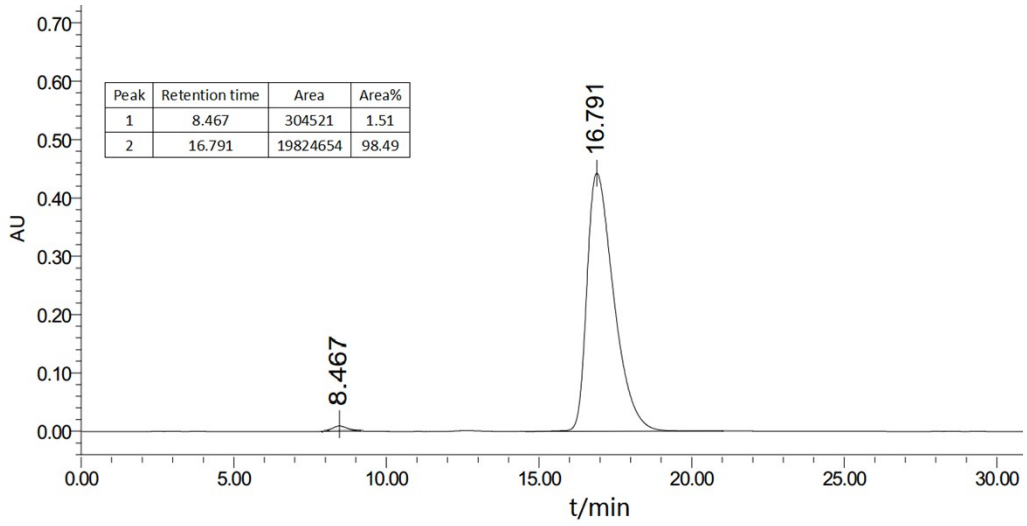


Fig. S17 HPLC chromatogram of compound **2**.

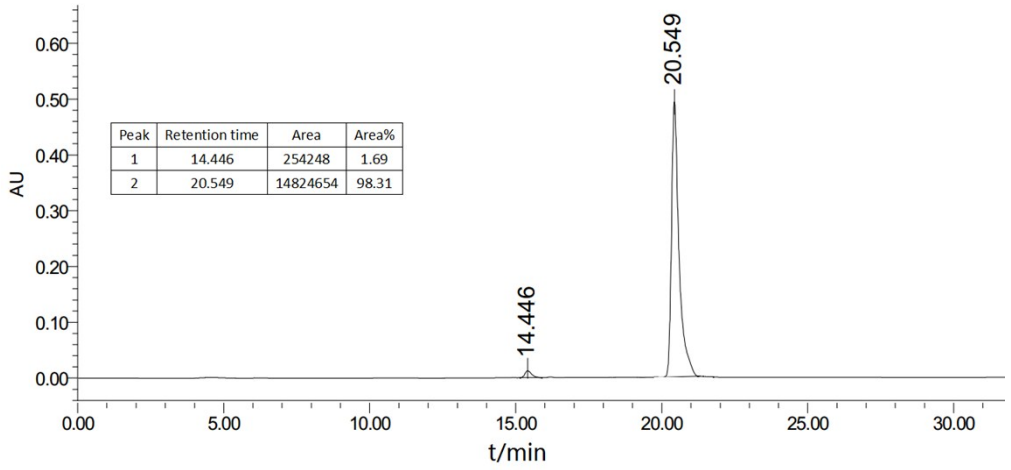


Fig. S18 HPLC chromatogram of **HMX-1**.

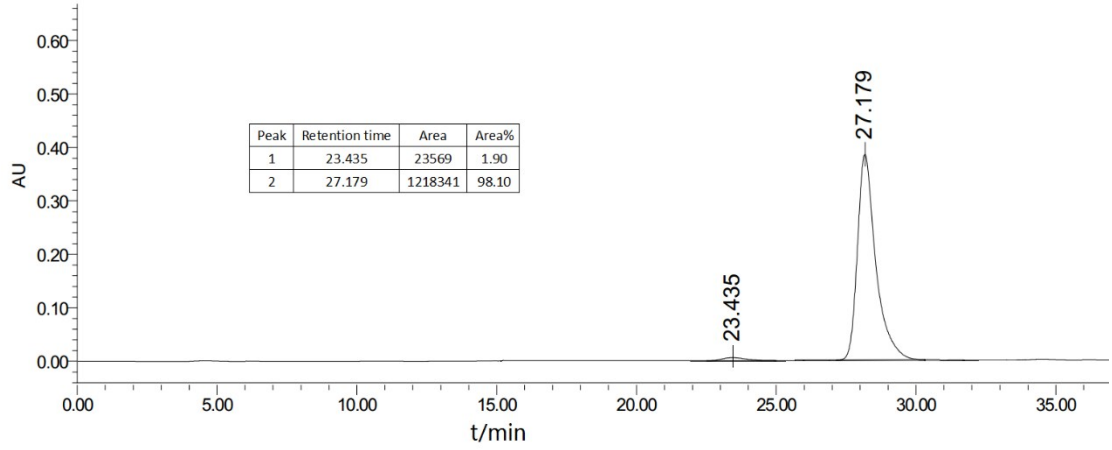


Fig. S19 HPLC chromatogram of **aniline nitrogen mustard hydrochloride**.