

**Supplementary Information**  
**for**  
**Indomethacin-guided cancer selective prodrug conjugate**  
**activated by histone deacetylase and tumour-associated protease**

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## 1. Experiment Section

### 1.1 Materials

4-Aminobutyric acid (Aldrich), Benzyl chloromate (TCI), *tert*-Buthanol (SAMSHUN), *N,N'*-Dicyclohexylcarbodiimide (DCC) (Aldrich), 4-Dimethylaminopyridine (DMAP) (TCI), Indomethacin (TCI), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Aldrich), trifluoroacetic acid (TCI), Lys(Ac)-OH (Bachem) Perchloric acid (TCI), *tert*-butyl bromoacetate (Alfa aesar), Doxorubicin (Carbosynth) 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) (Irish), *N,N'*-Diisopropylethylamine (DIPEA) (Aldrich) were received and used as received. Column chromatography was performed using silica gel 60 (70 ~ 230 mesh) as stationary phase. Analytical thin layer chromatography was performed using 60 silica gel (precoated sheets with 0.25 mm thickness). The mass spectra were obtained on an IonSpecHiResESI mass spectrometer. The <sup>1</sup>H and <sup>13</sup>C NMR spectra were collected on Varian 300 and 400 MHz spectrometers using CDCl<sub>3</sub>, DMSO-d<sub>6</sub>, and CD<sub>3</sub>OD with TMS used as an internal reference. Human cervical cancer cell line HeLa, HepG2 and Caco-2 cell lines were purchased from American Type Culture Collection (ATCC) (VA, USA). HCT 116 and MIA PaCa-2 cell lines were purchased from the KOREA cell bank (Seoul, KOR). All reagents for cell culture were purchased from Thermo Fisher Scientific Korea, Ltd., (Seoul, KOR). The 6 to 8 week old nude mice (BALB/c, nu/nu) were obtained from Orientbio Ltd., (Sunnam, KOR).

### 1.2 Synthesis

**Compounds 3-5** was synthesized by previously reported procedures.<sup>1</sup>

**Compound 7** was synthesized according to the previous report.<sup>2</sup>

**Synthesis of 6:** Indomethacin (737 mg, 2.06 mmol), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (640 mg, 4.12 mmol), 4-Dimethylaminopyridine (DMAP) (378 mg, 3.09 mmol) and compound **5** (410 mg, 2.57 mmol) were dissolved in *N,N'*-dimethylformamide (20 ml). The reaction mixture was stirred for overnight at room temperature. After the reaction was completed, the solvent was removed under reduced pressure and diluted with water, extracted ethyl acetate (3 X 10 ml). The organic layer was dried with anhydrous sodium sulfate and concentrated. The residue was purified by silica gel

column chromatography (5 % MeOH in DCM) to afford 912 mg (88.7 %) of compound **6**. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.68 (d, *J* = 8.32 Hz, 2H); 7.49 (d, *J* = 8.36 Hz, 2H); 6.88 (d, *J* = 9.48 Hz, 2H); 6.70 (dd, *J* = 9.04 Hz, 1H); 5.92 (s, 1H); 3.82 (s, 3H); 3.63 (s, 2H); 3.24 (m, 2H); 2.38 (s, 3H); 2.16 (m, 2H); 1.69 (m, 2H); 1.38 (s, 9H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): 172.79, 170.19, 168.53, 156.48, 139.70, 139.70, 136.52, 133.84, 131.40, 131.09, 130.54, 129.41, 115.37, 113.07, 112.59, 100.85, 80.78, 55.93, 39.30, 33.02, 32.45, 28.23, 24.87, 13.53 ppm. ESI-MS *m/z* (M + Na<sup>+</sup>): calcd 521.2, found 521.4.

**Synthesis of 2:** To a solution of compound **6** (910 mg, 1.82 mmol) in dichloromethane (10 ml) was added trifluoroacetic acid (2 ml) and mixture was stirred for overnight at room temperature. The solvent and an excess of TFA were removed by co-evaporation with toluene, leaving 731 mg of the compound **2** as a white powder. (90.7 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.69 (d, *J* = 8.40 Hz, 2H); 7.50 (d, *J* = 8.44 Hz, 2H); 7.11 (s, 1H); 6.95 (m, 2H); 6.67 (dd, *J* = 9.16 Hz, 1H); 3.81 (s, 3H); 3.56 (s, 2H); 3.23 (m, 2H); 2.33 (s, 3H); 2.26 (m, 2H); 1.76 (m, 2H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): 175.32, 170.49, 168.45, 156.25, 139.19, 136.03, 134.17, 131.36, 131.04, 131.00, 129.29, 115.14, 113.84, 111.94, 101.50, 55.88, 39.10, 32.15, 31.67, 24.83, 13.71 ppm. ESI-MS *m/z* (M – H<sup>+</sup>): calcd 441.1, found 441.1.

**Synthesis of 8:** Compound **2** (100 mg, 0.23 mmol), EDC (54 mg, 0.34 mmol), DMAP (56 mg, 0.46 mmol) and compound **7** (84 mg, 0.34 mmol) were dissolved in *N,N'*-dimethylformamide (10 ml). The reaction mixture was stirred for overnight at room temperature. After completion of reaction, the reaction mixture was evaporated under reduced pressure to remove the excess solvent, diluted with water and extracted ethyl acetate (3 X 100 ml). The organic layer was dried with anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by silica gel column chromatography (10 % MeOH in DCM) to afford 93.6 mg (60.8 %) of compound **8**. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.68 (d, *J* = 8.60 Hz, 2H); 7.49 (d, *J* = 8.56 Hz, 2H); 6.91 (m, 2H); 6.70 (dd, *J* = 9.02 Hz, 1H); 6.60 (s, 1H); 6.40 (s, 1H); 6.37 (s, 1H); 6.13 (s, 1H); 4.31 (m, 1H); 3.81 (s, 3H); 3.62 (s, 2H); 3.27 (m, 2H); 3.20 (m, 2H); 2.37 (s, 3H); 2.18 (m, 2H); 1.94 (s, 3H); 1.75 (m, 4H); 1.61 (m, 2H); 1.44 (s, 9H); 1.33 (m, 2H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): 172.60, 171.87, 170.78, 170.62, 168.57, 156.40, 139.69, 136.56, 133.87, 131.42, 131.17, 130.64, 129.40, 115.35, 113.09, 112.25, 101.23, 82.22, 55.99, 52.66, 39.19, 33.58, 32.42, 32.03, 29.89, 29.05, 28.19, 25.48, 23.41, 22.63, 13.57 ppm. ESI-MS *m/z* (M + Na<sup>+</sup>): calcd 691.3, found 691.4

**Synthesis of 9:** To a solution of compound **8** (93 mg, 0.14 mmol) in dichloromethane (5 ml) was added trifluoroacetic acid (1 ml) and mixture was stirred at room temperature overnight. The solvent and excess of TFA were removed by co-evaporation with toluene, leaving 68 mg of the compound **9** as a yellow powder. (79.1 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.68 (d, *J* = 8.36 Hz, 2H); 7.49 (d, *J* = 8.24 Hz, 2H); 6.95 (d, *J* = 2.20 Hz, 1H); 6.86 (d, *J* = 8.96 Hz, 1H); 6.67 (dd, *J* = 8.76 Hz, 1H); 4.17 (t, *J* = 7.76 Hz, 1H); 3.81 (s, 3H); 3.59 (s, 2H); 3.36 (m, 2H); 3.22 (m, 2H); 2.36 (s, 3H); 2.20 (t, *J* = 6.20 Hz, 2H); 1.92 (s, 3H); 1.72 (m, 2H); 1.59 (m, 2H); 1.34 (m, 2H); 1.31 (m, 2H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): 173.45, 171.82, 171.57, 168.81, 156.17, 156.15, 139.40, 136.46, 133.76, 131.30, 131.07, 130.78, 129.30, 115.10, 113.17, 111.68, 101.52, 76.99, 55.79, 48.43, 39.09, 38.97, 33.29, 31.85, 31.77, 28.71, 25.39, 22.81, 22.55, 13.26 ppm. ESI-MS *m/z* (*M* + H<sup>+</sup>): calcd 613.24, found 613.4, *m/z* (*M* + Na<sup>+</sup>): calcd 635.2, found 635.4

**Synthesis of 1:** Compound **9** (250 mg, 0.41 mmol), 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU) (189 mg, 0.49 mmol), *N,N*-Diisopropylethylamine (DIPEA) (64 mg, 0.49 mmol) and doxorubicin (288 mg, 0.49 mmol) were dissolved in *N,N*'-dimethylformamide (20 ml). The reaction mixture was stirred at room temperature overnight. After the reaction was completed, the reaction mixture was concentrated under reduced pressure, diluted with water and extracted ethyl acetate (3 X 100 ml). The organic layer was dried with anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by HPLC to afford 110 mg (23.8 %) of compound **1**. <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.96 (m, 1H); 7.76 (m, 1H); 7.66 (d, *J* = 9.68 Hz, 2H); 7.48 (d, *J* = 8.28 Hz, 2H); 7.38 (d, *J* = 8.36 Hz, 1H); 6.89 (s, 1H); 6.83 (m, 2H); 6.66 (m, 1H); 6.33 (br, 1H); 6.15 (br, 1H); 5.99 (br, 1H); 5.54 (br, 1H); 5.19 (br, 1H); 4.75 (s, 2H); 4.66 (br, 1H); 4.26 (br, 1H); 4.17 (br, 1H); 4.05 (br, 3H); 3.97 (s, 3H); 3.78 (s, 3H); 3.69 (s, 1H); 3.58 (m, 2H); 3.48 (s, 1H); 3.25 (m, 2H); 3.13 (m, 4H); 2.95 (m, 1H); 2.32 (s, 3H); 2.15 (m, 4H); 1.94 (br, 2H); 1.89 (s, 3H); 1.59 (br, 6H); 1.42 (br, 2H); 1.27 (m, 3H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): 214.14, 186.77, 186.55, 173.00, 172.41, 171.19, 171.05, 168.58, 161.15, 160.94, 156.30, 155.55, 139.70, 136.64, 135.84, 135.56, 135.38, 134.17, 133.77, 131.42, 131.38, 131.09, 130.70, 130.67, 129.40, 120.74, 119.86, 118.55, 115.26, 113.09, 112.99, 111.90, 111.51, 101.54, 100.99, 69.38, 68.84, 68.04, 67.75, 65.69, 56.61, 56.01, 55.98, 53.27, 39.01, 38.88, 35.79, 34.09, 33.88, 33.24, 32.21, 29.35, 25.57, 25.22, 23.35, 22.67, 17.14, 13.58 ppm. ESI-MS *m/z*

(M – 2H<sup>+</sup>): calcd 1136.4, found 1136.4, m/z (M + 2H<sub>2</sub>O – H<sup>+</sup>): calcd 1172.4, found 1172.4.

### 1.3 UV/Vis and Fluorescence Spectroscopy

All fluorescence and UV/Vis absorption spectra were recorded in RF-5301PC and S-3100 spectrophotometer, respectively. Fluorescence measurements were performed by commercially standard HDAC (cayman) and Trypsin (welgene). Stock solutions (1 mM) of probe **1** were prepared in DMSO. 5 μM solutions of **1** in HDAC buffer were incubated with HDAC1 positive control for 90 min. Next, to the HDAC treated compound **1** were added trypsin and incubated for 0 to 24 hours. Excitation was carried out at 499 nm with excitation slit widths is 5, that of emission is 5 nm.

### 1.4 Cell culture

HepG2 and HCT 116 cells were cultured in RPMI medium 1640. HeLa and MIA PaCa-2 cells were cultured in Dulbecco Modified Eagle Medium (DMEM). Caco-2 cells were cultured in Minimum Essential Media (MEM). All media were supplemented with 10 % (v/v) FBS, penicillin (100 units/mL), and streptomycin (100 μg /ml) under 5 % (v/v) CO<sub>2</sub> and 95 % (v/v) humidity at 37 °C.

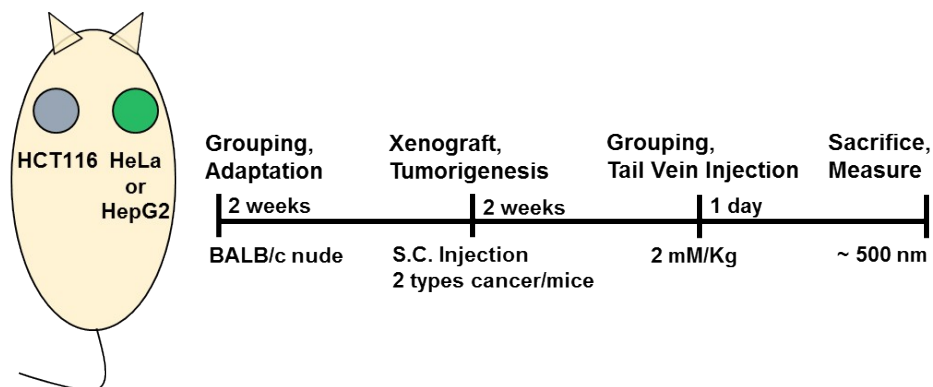
### 1.5 Confocal microscopy imaging

One day before imaging, the cells were seeded on Coverglass Bottom Dish (SPL Lifesciences Co., Ltd.) which was incubated in a humidified atmosphere containing 5 % (v/v) CO<sub>2</sub> at 37 °C. Cell images were obtained using confocal laser scanning microscopy (Zeiss LSM 510, Zeiss, Oberko, Germany). Other information is available in the figure captions.

### 1.6 *Ex vivo* fluorescence experiment

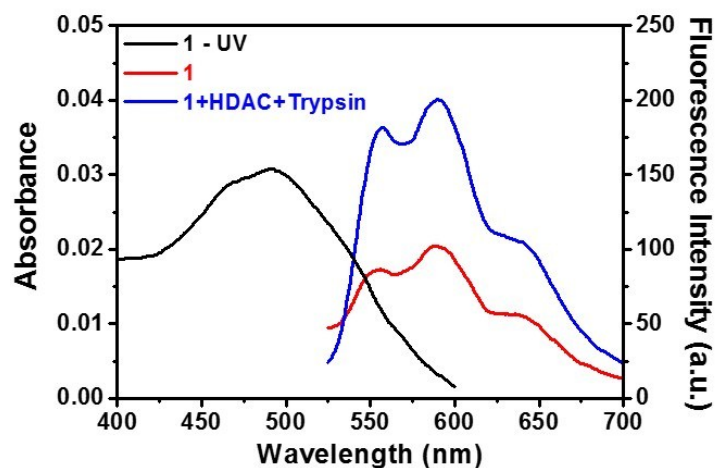
Exponentially growing HCT116, HeLa and HepG2 cells were harvested, adjusted to 4 × 10<sup>6</sup> cells/100 μl and injected subcutaneously into the right and left axilla of each nude mouse, respectively. 2-weeks after inoculation, the mice were randomly divided into two groups (6 mice/group) as follows HCT116/Hela and HCT116/HepG2, respectively. Each group were

administrated with **1** by tail vein injection at 2 mM/kg concentration for 1 day.

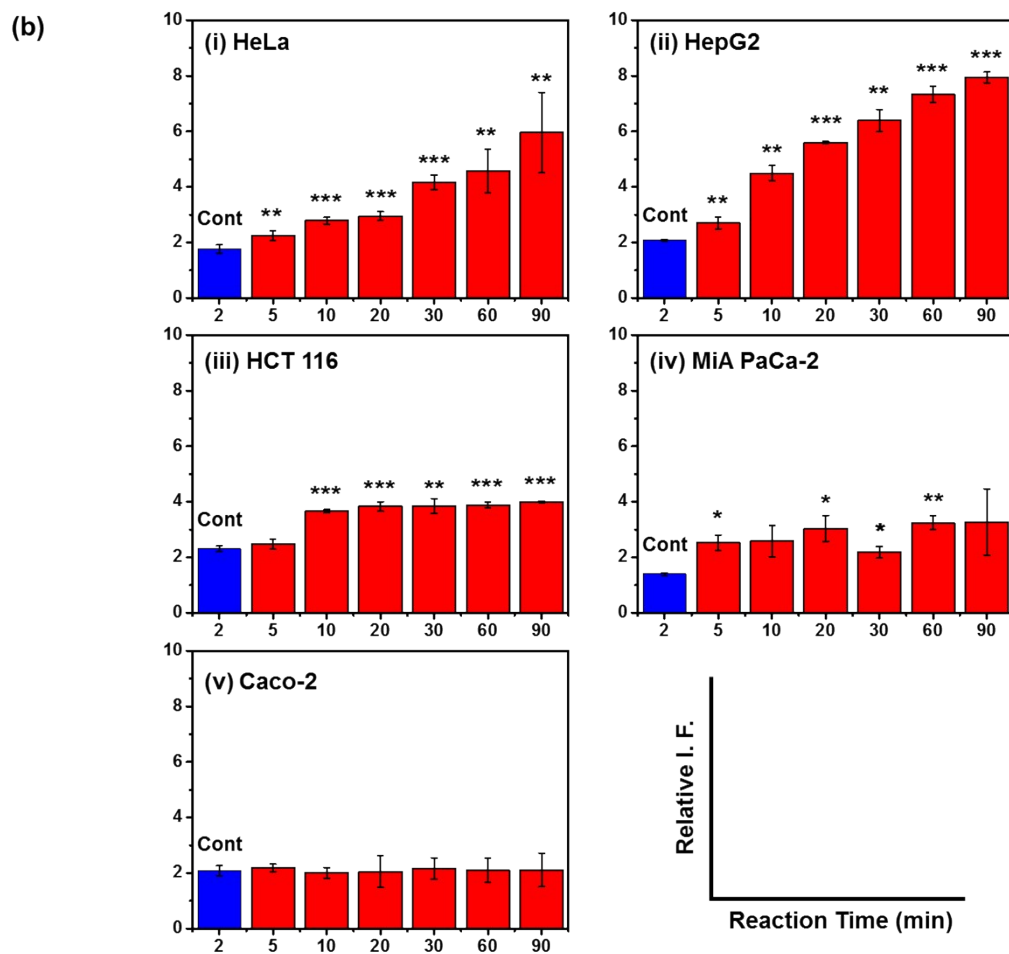
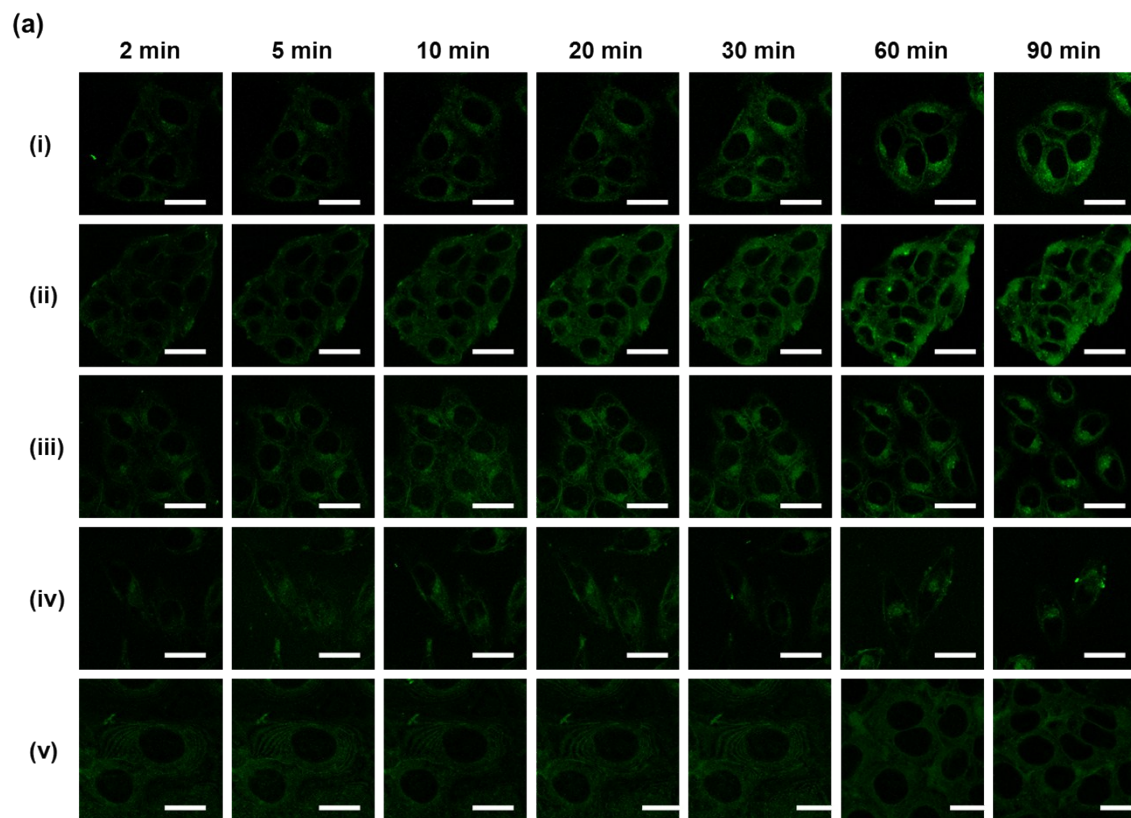


**Figure S1.** Graphical presentation of *ex vivo* fluorescence experiment method.

## 2. Characterization spectra and other supporting studies



**Figure S2.** UV-visible and fluorescence spectrum of **1** and treated HDAC and trypsin.

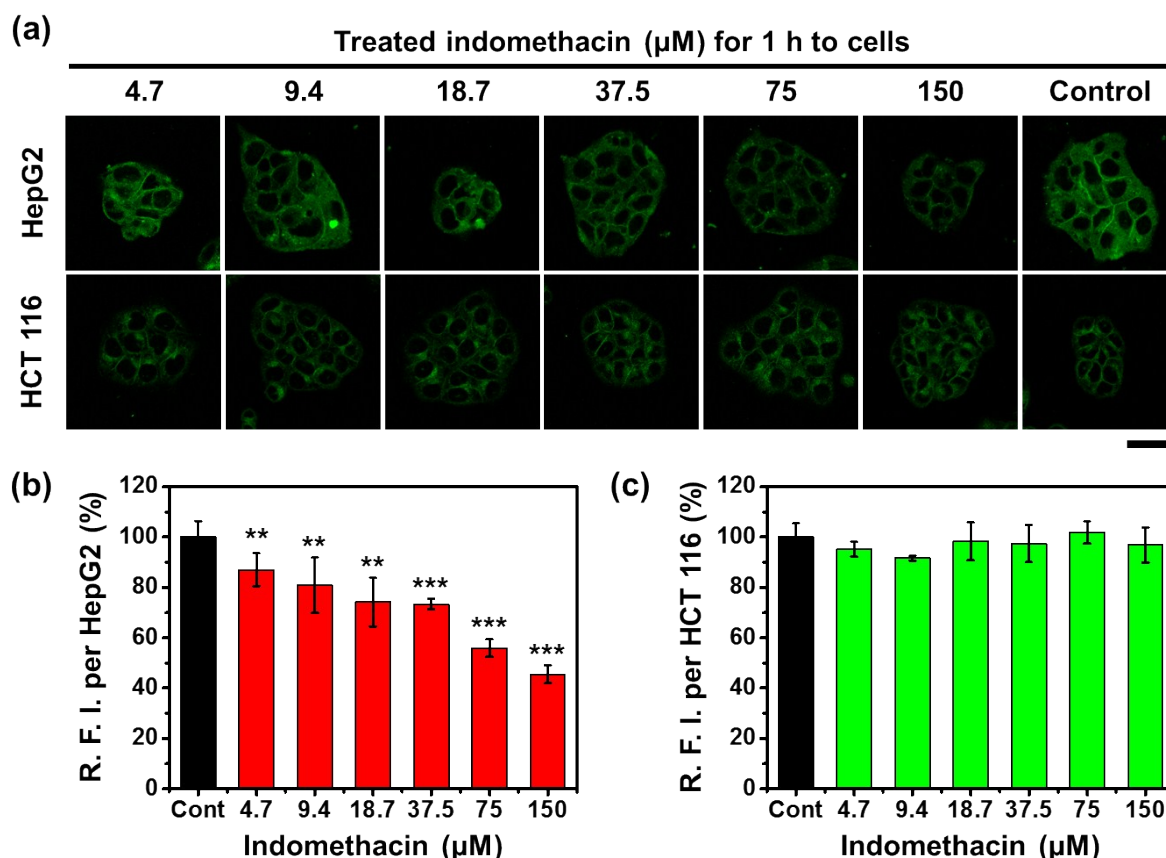


**Figure S3.** (a) Confocal and fluorescence microscopy images of **1** depend on incubation time (2, 5, 10, 20, 30, 60 and 90 min). Cells were treated with probe **1** (5  $\mu$ M) and incubated in 5 % (v/v) CO<sub>2</sub> and 95 % (v/v) humidity at 37 °C. Confocal microscopy images were obtained from which excitation wavelength was 488 nm (Laser power 10 %) and filter was long pass 505 nm and detector gain value was 850. (i) HeLa; (ii) HepG2; (iii) HCT 119; (iv) MIA PaCa-2; (v) Caco-2. The scale bars indicate 30  $\mu$ m. (b) Histogram of relative fluorescence intensity of (i) HeLa; (ii) HepG2; (iii) HCT 119; (iv) MIA PaCa-2; (v) Caco-2 depend on incubation time were represented using image J program. Results represent the mean ( $\pm$ SEM) of three independent experiments (n=3). The statistical signification was marked as \*, \*\* and \*\*\* for  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  respectively, compared with the control.

Cell line	Origin	Enzyme Activity		
		HDAC	CTSL	COX-2
HeLa	Cervical cancer	+	+	+
HepG2	Liver cancer	+	+	+
HCT 116	Colon cancer	+	+	-
MIA PaCa-2	Pancreatic cancer	+	+	-
Caco-2	Colon cancer	-	-	-

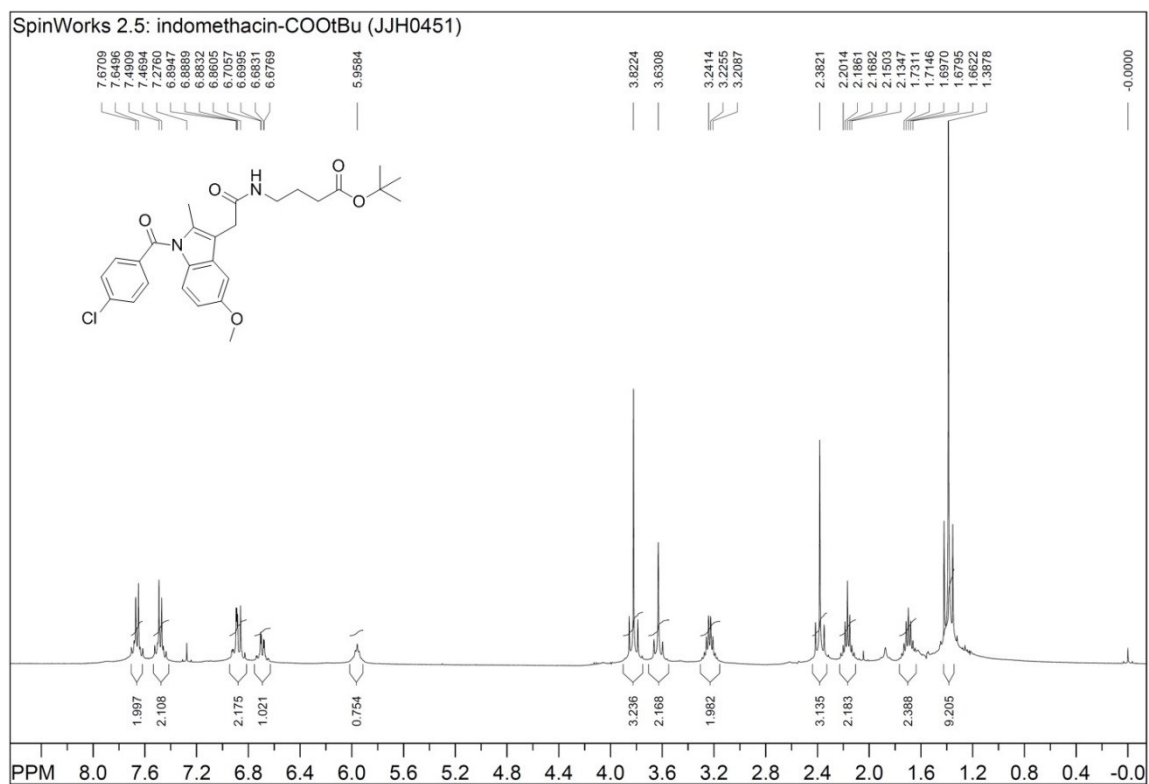
**Table S1.** Table of enzyme activity of each cell lines (HeLa, HepG2, HCT 116, MIA PaCa-2, Caco-2).



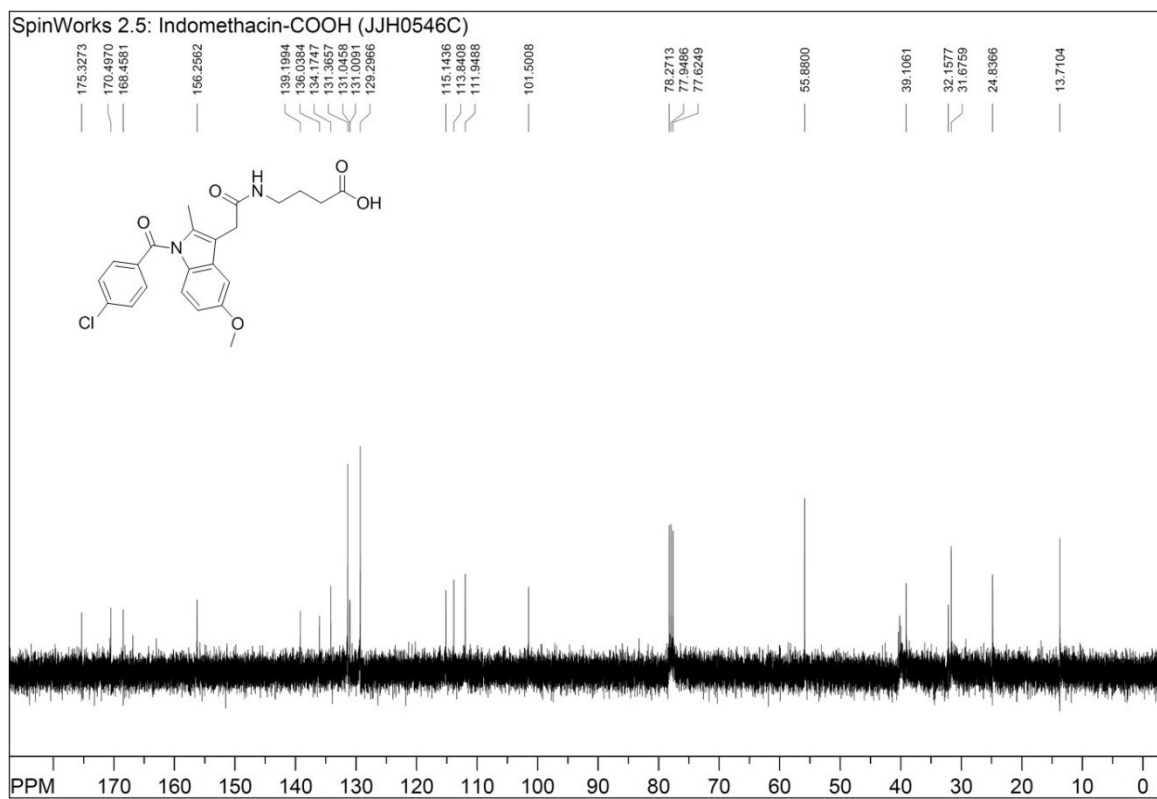


**Figure S4.** (a) Confocal microscopy images of **1** depending on the COX-2 inhibitor; indomethacin. Cell lines were treated with various concentrations of indomethacin ( $\mu\text{M}$ ) for 1 h in an incubator. Collected media were treated before **1** ( $5 \mu\text{M}$ ) was dissolved in them, and then they were incubated for 15 min. Control images show the cell line untreated with indomethacin. The scale bar indicates  $30 \mu\text{m}$ . Histograms of the relative fluorescence intensity per cell of (b) HepG2 cells and (c) HCT 116 depending on the varying concentration of indomethacin were represented using an image J program. Results represent the mean ( $\pm\text{SEM}$ ) of five independent experiments ( $n = 5$ ). The statistical signification was marked as \*\* and \*\*\* for  $p < 0.01$  and  $p < 0.001$  respectively, compared with the control.

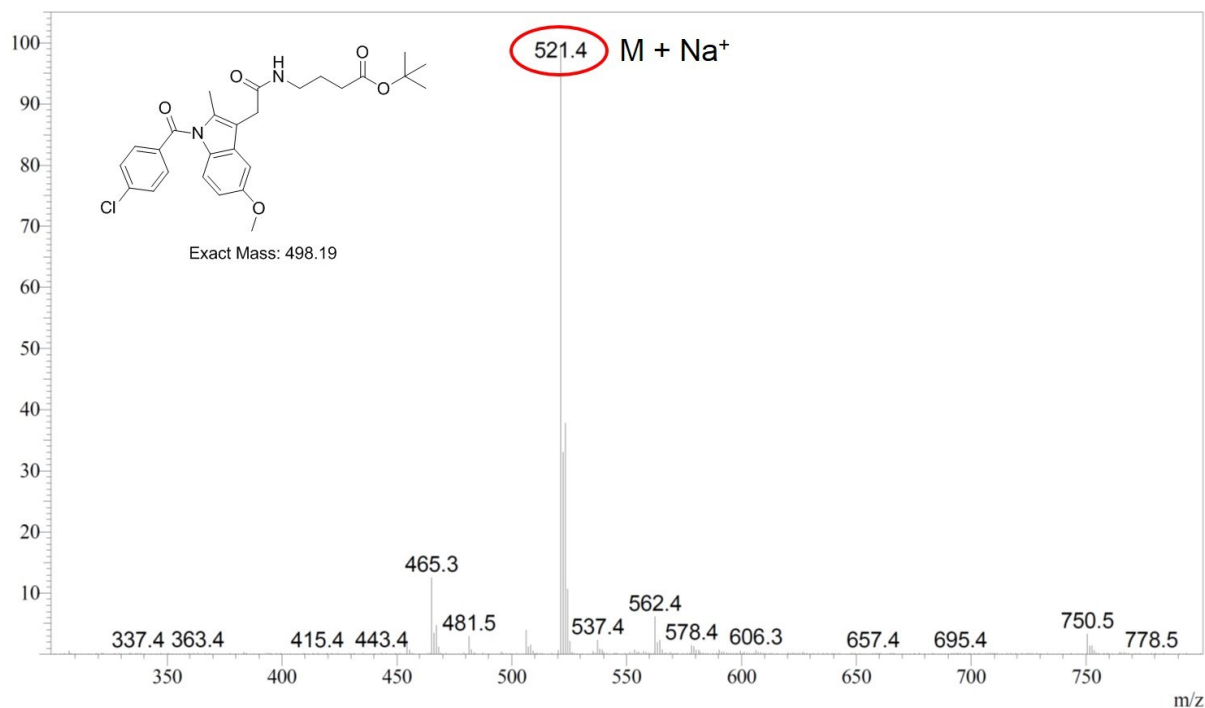
# <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectra



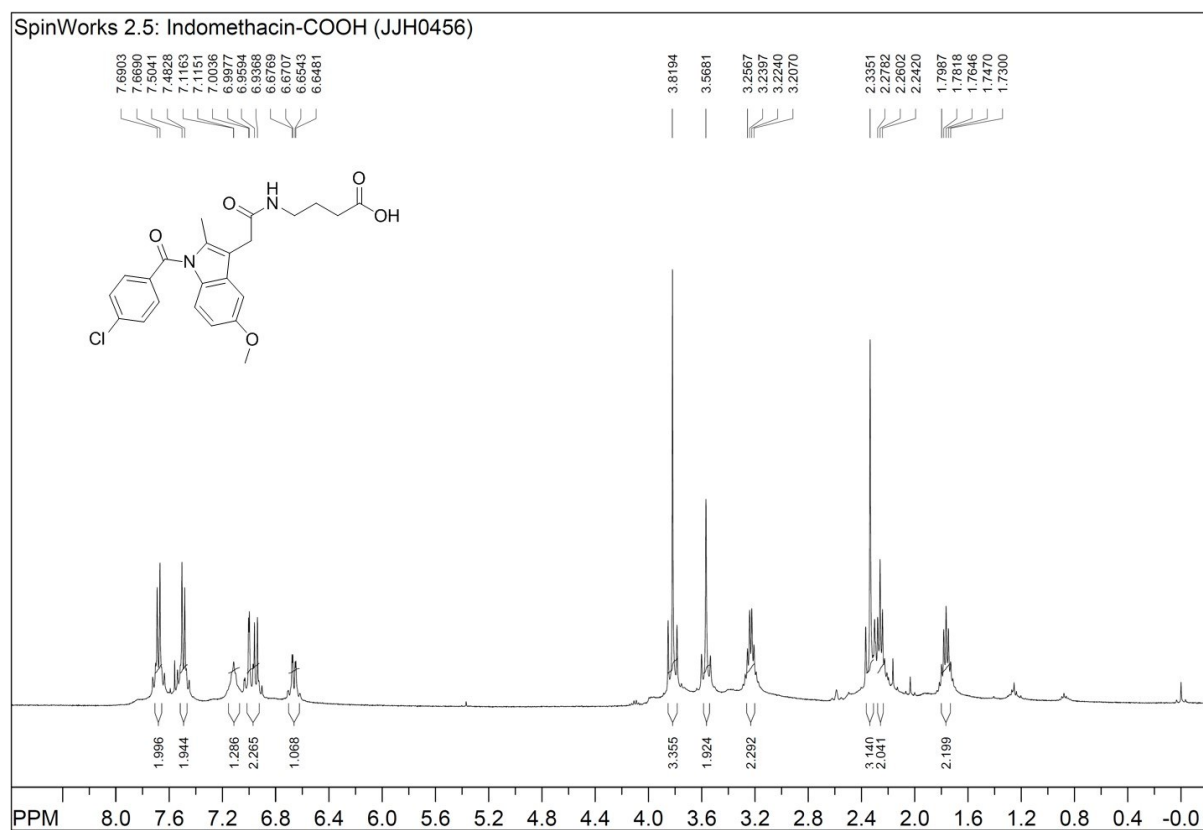
**Figure S5.** <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) spectrum of **6**.



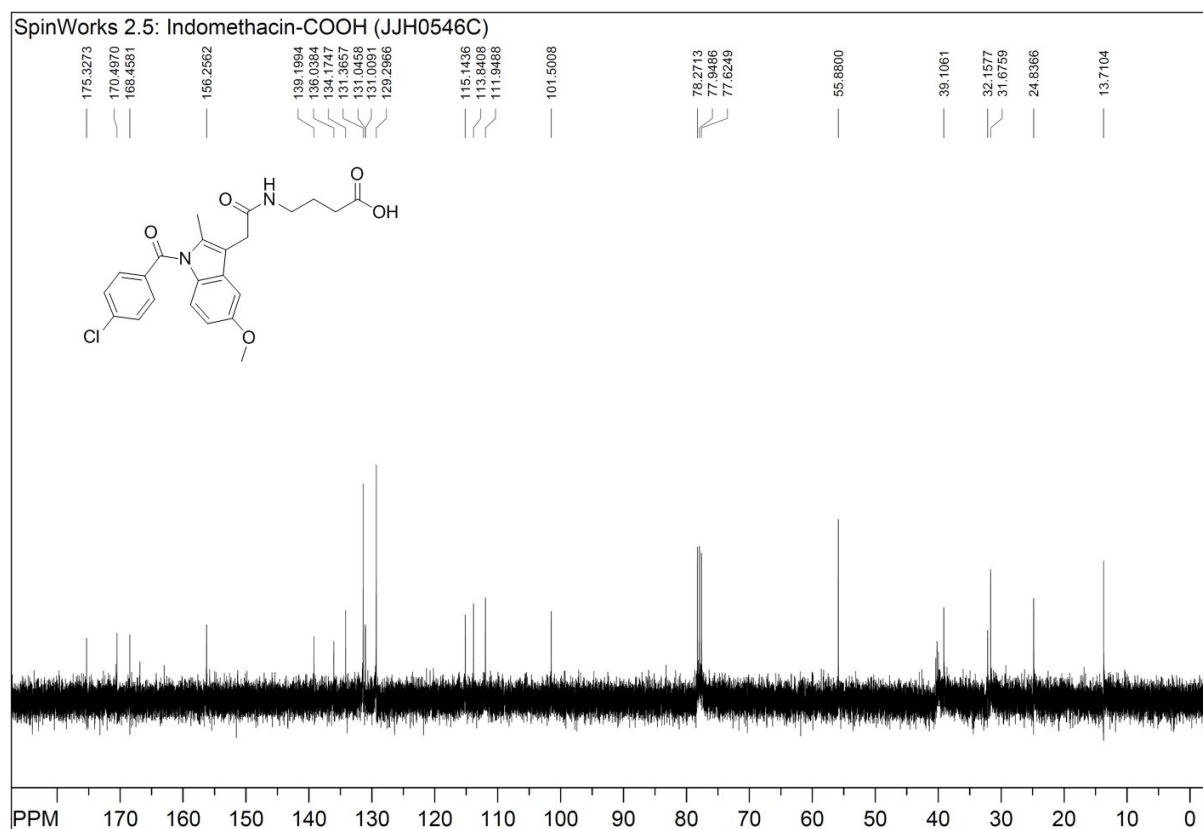
**Figure S6.**  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 100 MHz) spectrum of **6**.



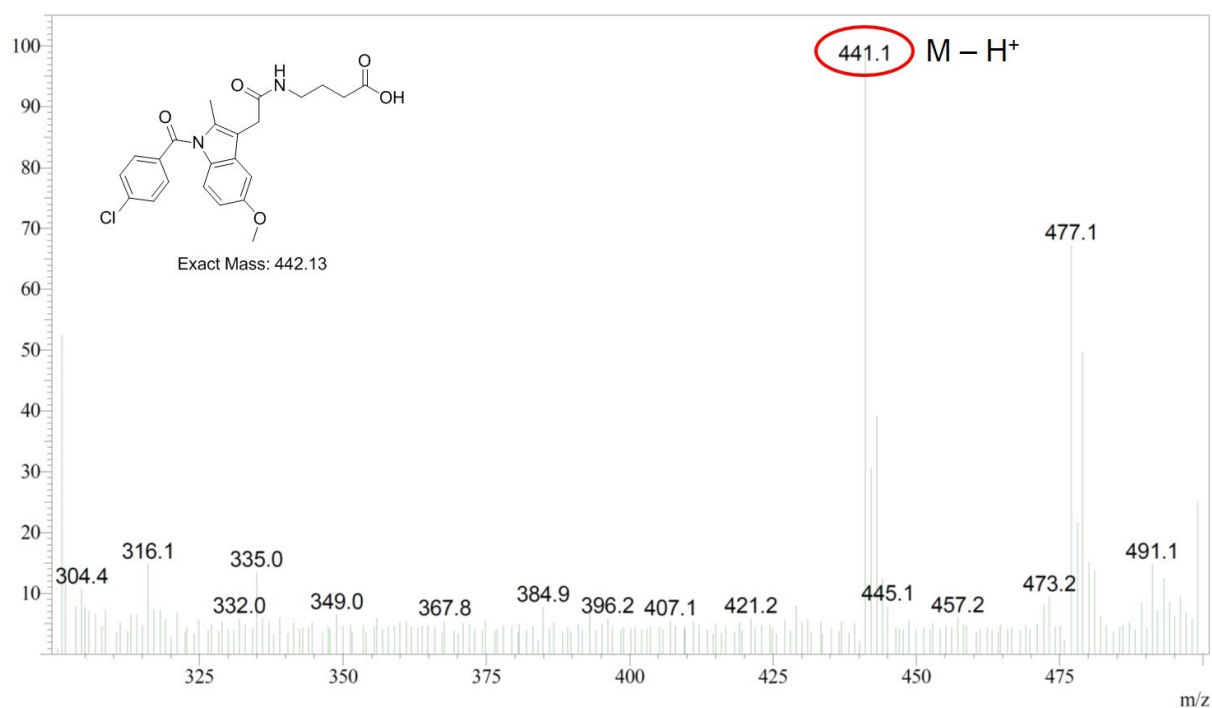
**Figure S7.** ESI-MS spectrum of **6**.



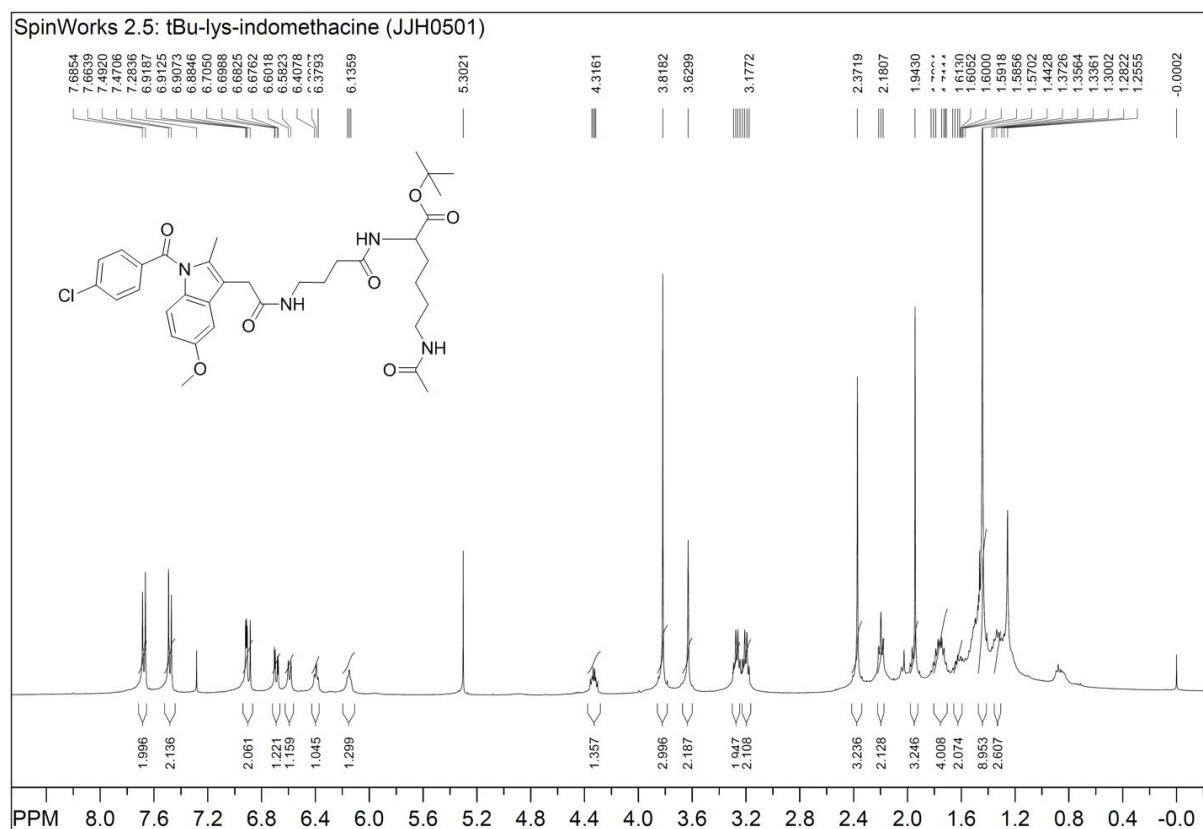
**Figure S8.**  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz) spectrum of **2**.



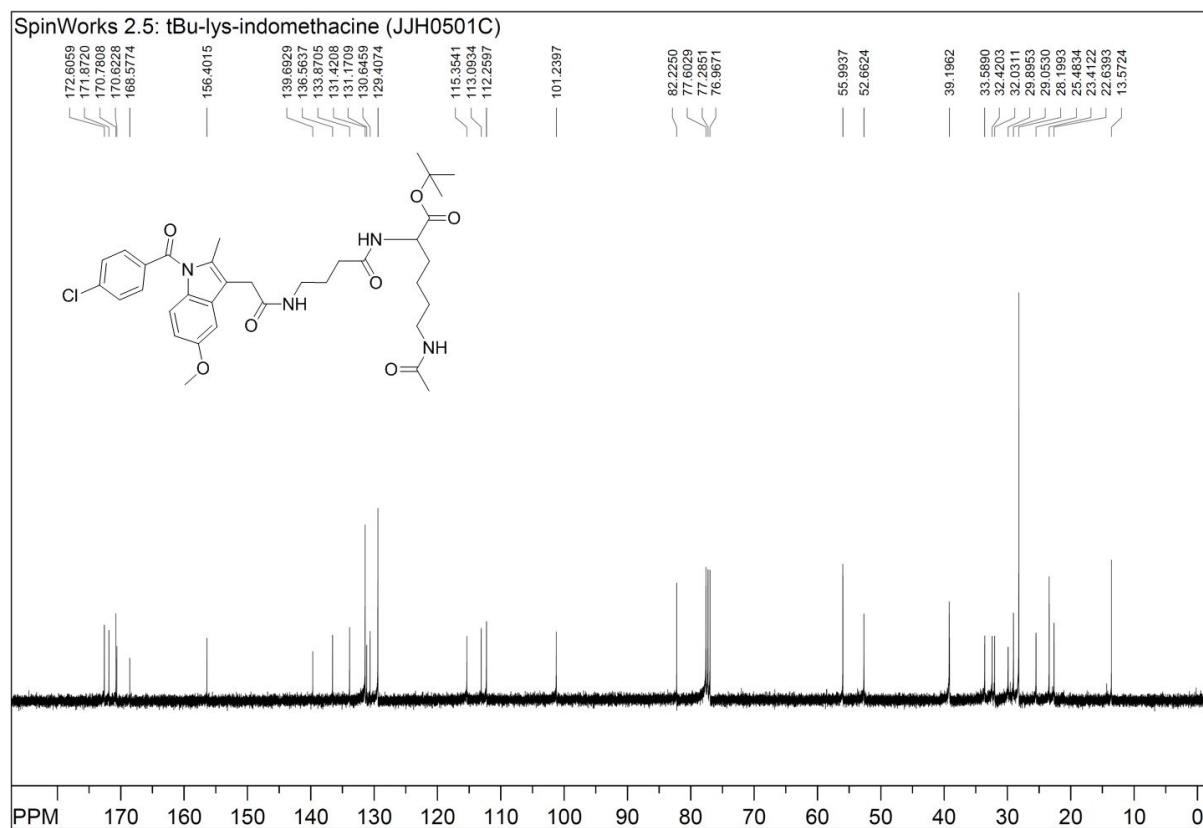
**Figure S9.** <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz) spectrum of **2**.



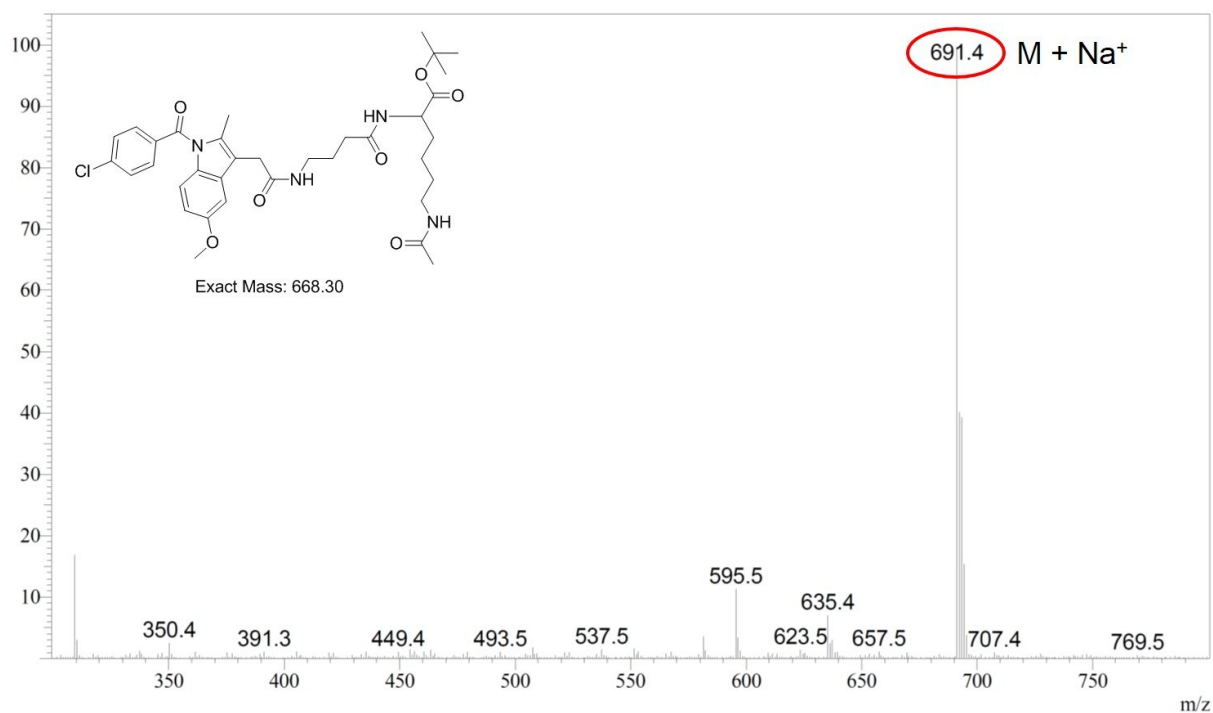
**Figure S10.** ESI-MS spectrum of **6**.



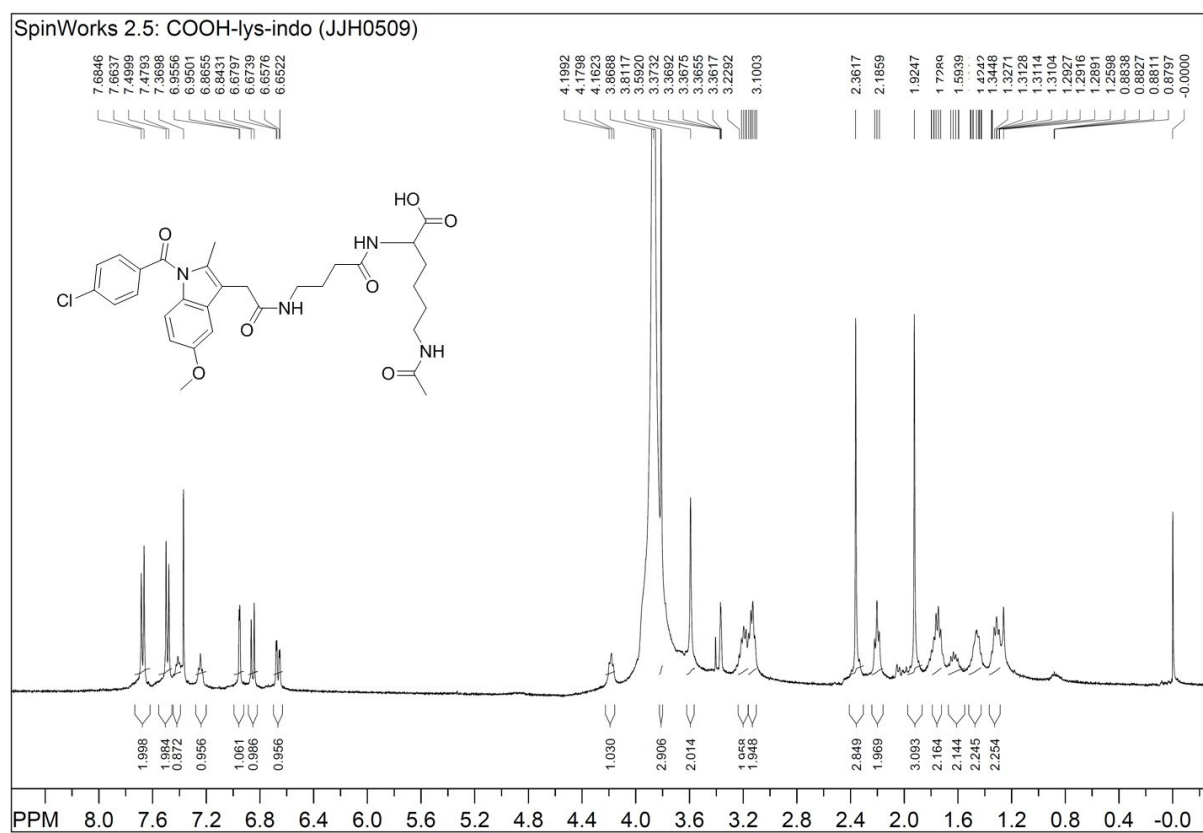
**Figure S11.** <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) spectrum of **8**.



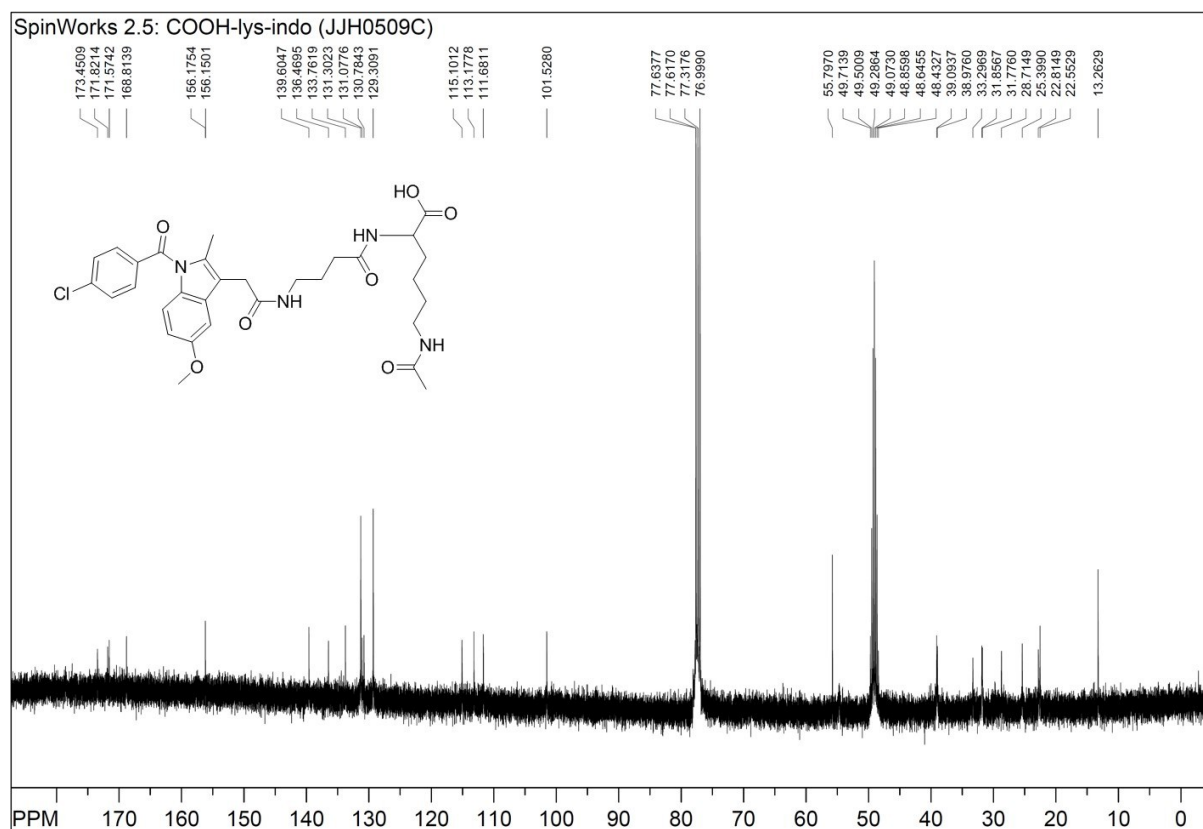
**Figure S12.** <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz) spectrum of **8**.



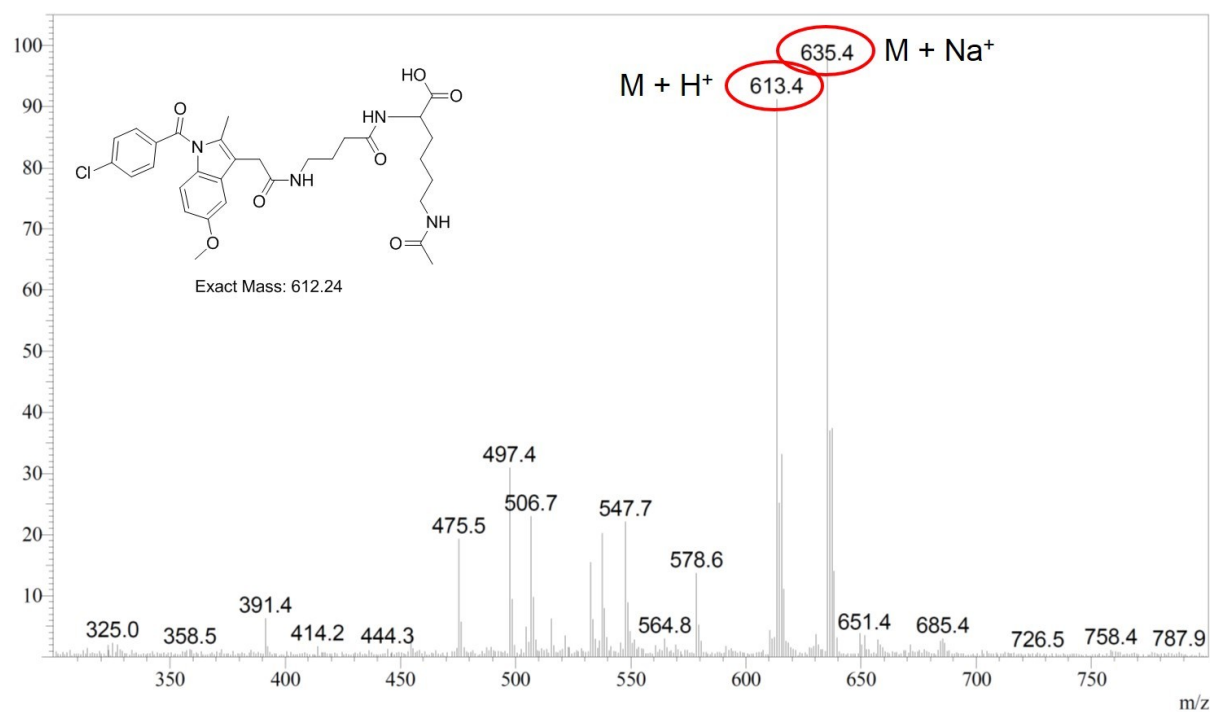
**Figure S13.** ESI-MS spectrum of **8**.



**Figure S14.** <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) spectrum of **9**.

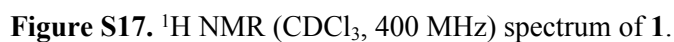


**Figure S15.** <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz) spectrum of **9**.

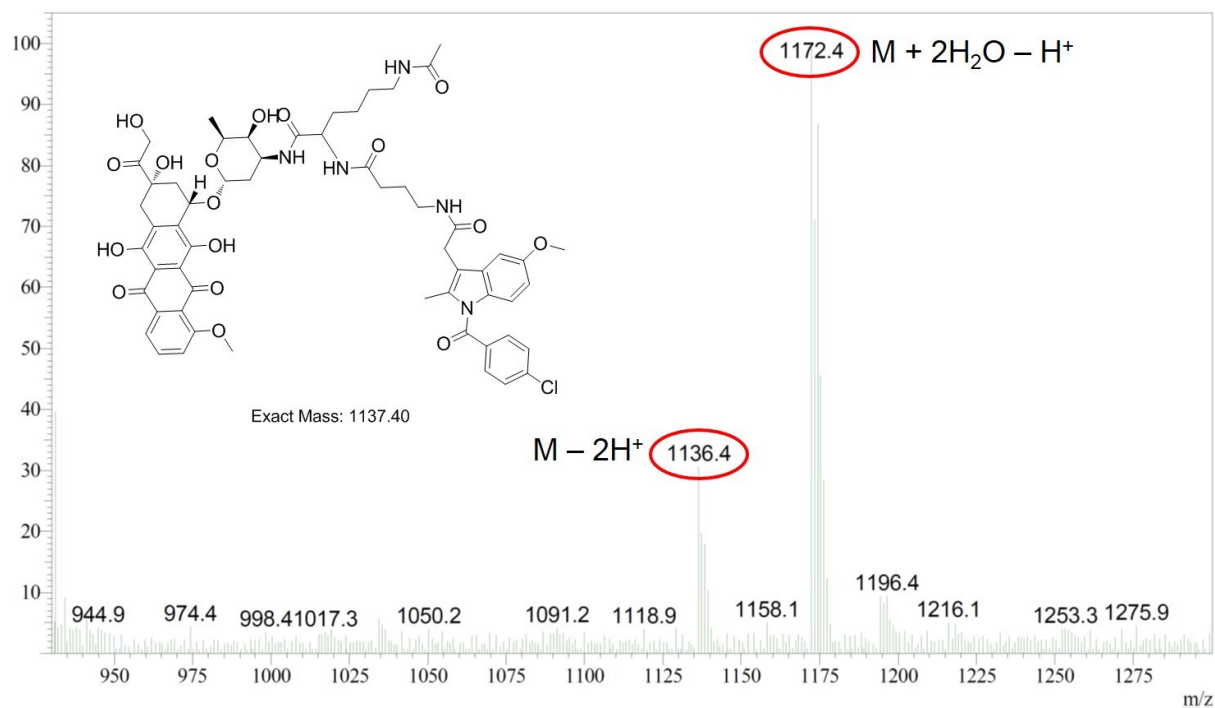


**Figure S16.** ESI-MS spectrum of **9**.

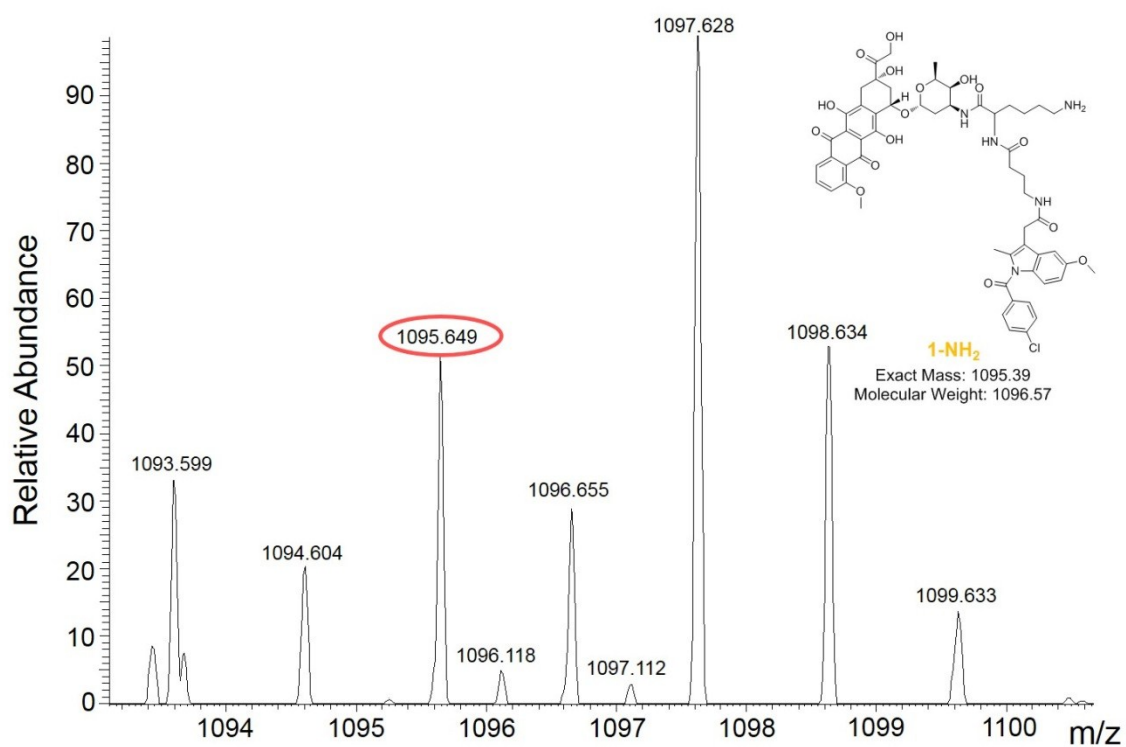




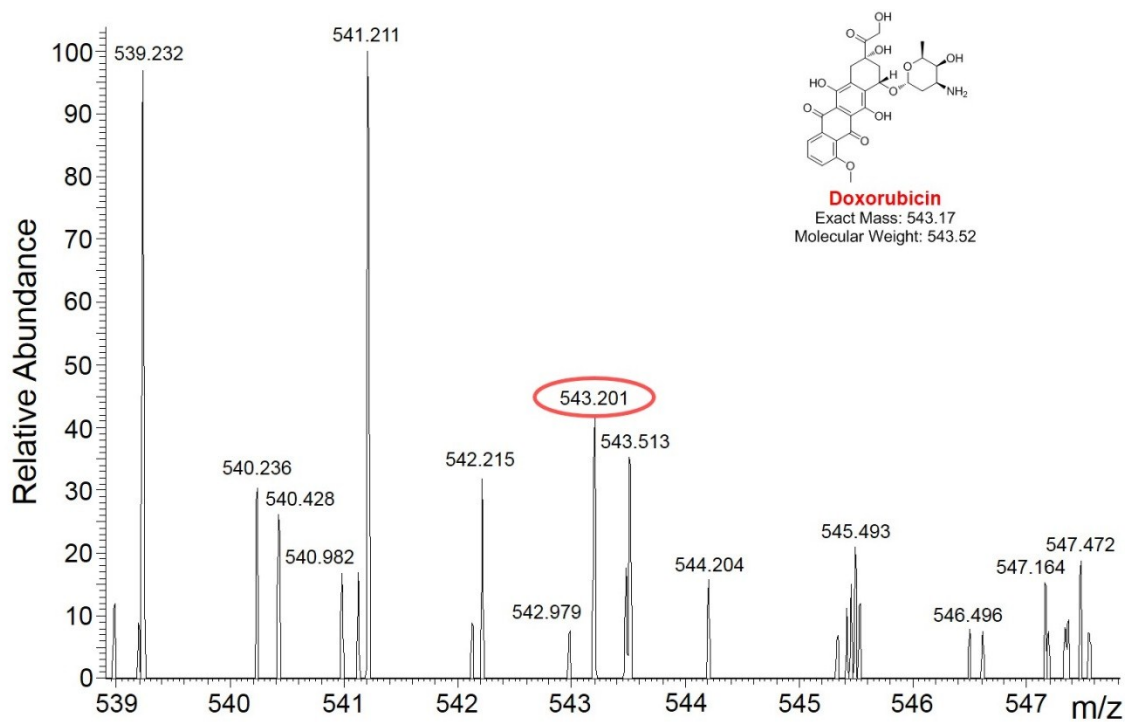




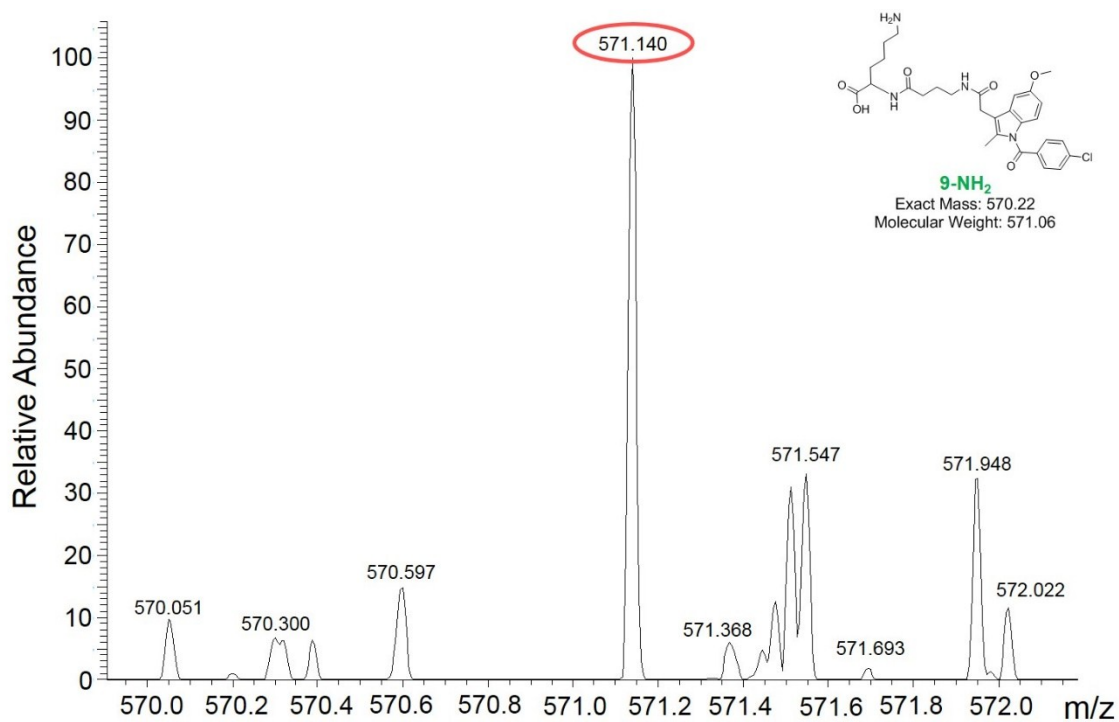
**Figure S19.** ESI-MS spectrum of **1**.



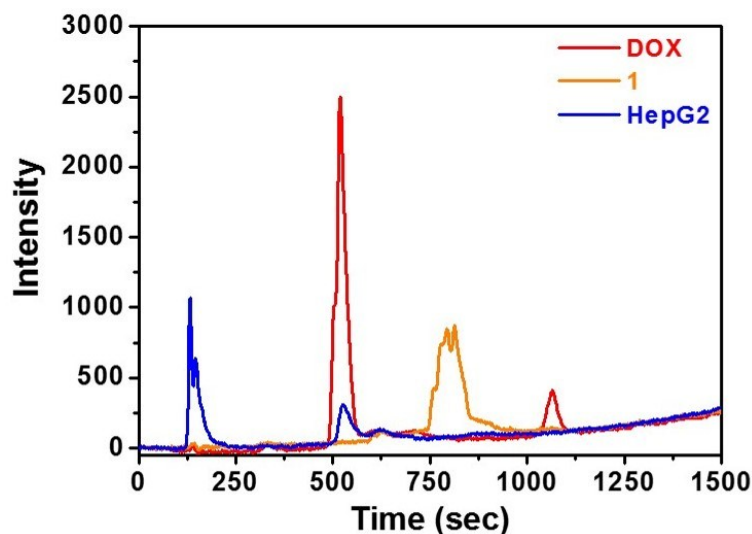
**Figure S20.** LTQ-Orbitrap-MS spectrum of compound **1** treated with HDAC.



**Figure S21.** LTQ-Orbitrap-MS spectrum of compound 1 treated with HDAC and trypsin.



**Figure S22.** LTQ-Orbitrap-MS spectrum of compound 1 treated with HDAC and trypsin.



**Figure S23.** HPLC data of free doxorubicin (red), probe **1** (orange), HepG2 cell extraction sample (blue). Condition: 60 % B during 2 min, then 60 % B to 100 % B for 23 min (A: 0.1 % TFA in water, B: MeOH) at 480 nm

### 3. Reference

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