A novel nitroreductase-enhanced MRI contrast agent and its potential application in bacterial imaging

Yun Liu[a,b], Leilei Zhang[c,d,*], Marc Nazare[e], Qingqiang Yao[b,*], Haiyu Hu[c,d,*]

[a]*School of Medicine and Life Sciences, University of Jinan-Shandong Academy of Medical Sciences, Jinan 250200, China*

[b]*Institute of Materia Medica, Shandong Academy of Medical Sciences, Key Laboratory for Biotech-Drugs Ministry of Health, Key Laboratory for Rare & Uncommol/Lon Diseases of Shandong Province, Jinan 250062, China*

[c]*State Key Laboratory of Bioactive Substances and Function of Natural Medicine, Institute of Materia Medica, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100050, China*

[d]*Beijing Key Laboratory of Active Substances Discovery and Drugability Evaluation, Institute of Materia Medica, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100050, China*

[e]*Leibniz-Forschngsinstitut für Molekulare Pharmakologie (FMP), Campus Berlin-Buch, 13125 Berlin, Germany*

*Corresponding authors.

E-mail addresses: zhangleilei@immol/L.ac.cn (Leilei Zhang), yao_immol/L@163.com (Qingqiang Yao), haiyu.hu@immol/L.ac.cn (Haiyu Hu)

Contents

Abbreviations

ATCC = American Type Culture Collection

DCM = Dichloromethane

DMA = Dimethylacetamide

DMAP = 4-Dimethylaminopyridine

eq. = equivalents

ESI-MS = Electrospray Ionisation Mass Spectrometry

HPLC = High Performance Liquid Chromatography

HRMS = High Resolution Mass Spectrometry

LC–MS = Liquid Chromatography Mass Spectrometry

MTS = 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium

NMR = Nuclear Magnetic Resonance

OD = Optical Density

PBS = Phosphate Buffered Saline

r.t. = room temperature

TFA = Trifluoroacetic Acid

1. Synthesis

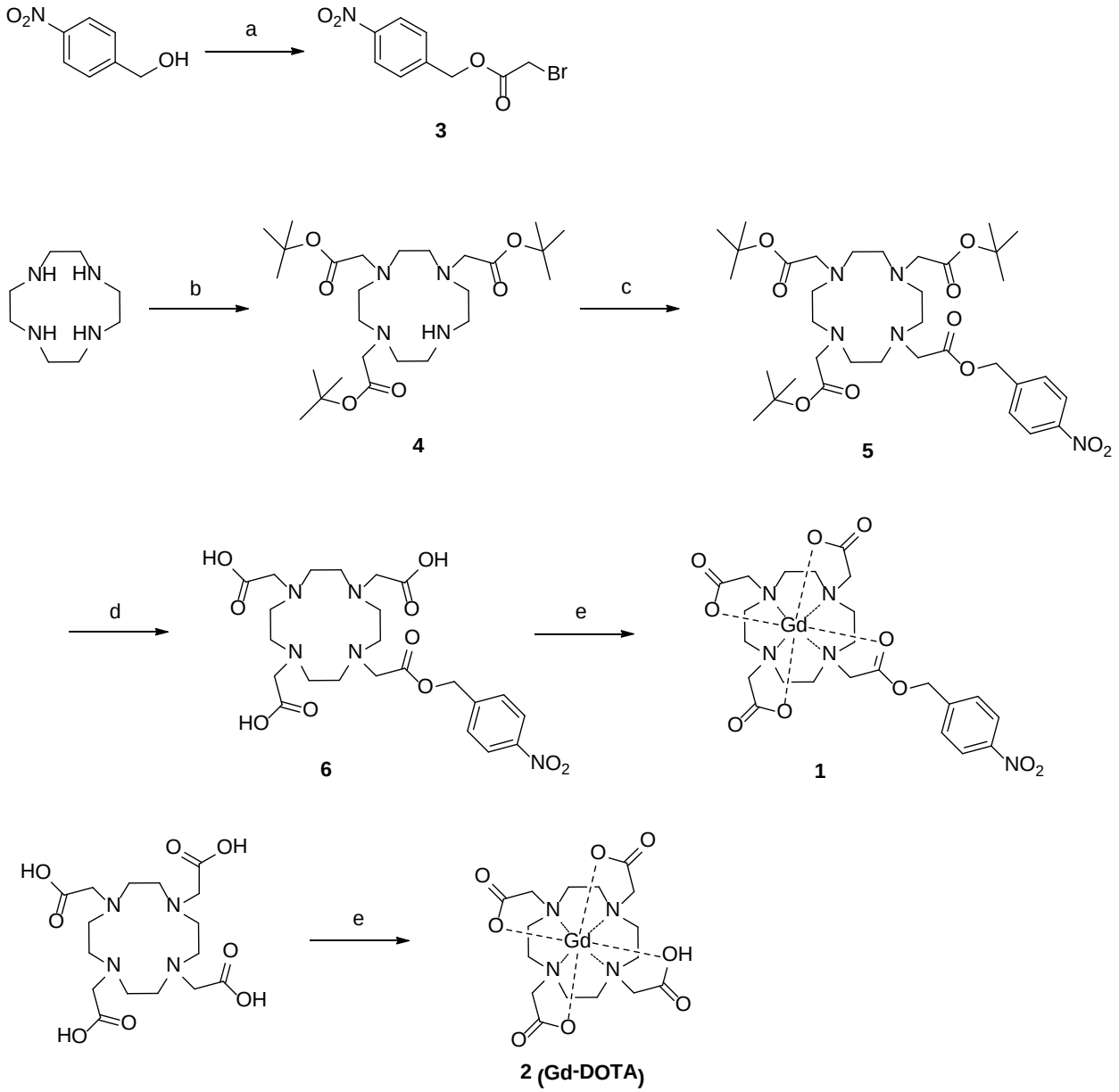


Figure 1 Synthesis of probe **1**. (a) bromoacetyl bromide, DMAP, toluene, 92%. (b) *t*-Butyl bromoacetate, sodium acetate, DMA, 62%. (c) Compound **3**, K_2CO_3, MeCN, 89%. (d) TFA/ trimethylsilane/H_2O, 0 °C, 63%. (e) $GdCl_3 \cdot 6H_2O$, NaOH, pH 6.5-7.0, 61%.

General methods

All chemicals were purchased from J&K. Commercially available reagents were used without further purification. Unless otherwise noted, all reactions were performed under a nitrogen or argon

atmosphere. Thin layer chromatography (TLC) was carried out with Silica Gel 60 F254, and column chromatography with silica gel (200–300 mesh). All ^{1}H NMR spectra were recorded at 600 MHz, ^{13}C NMR spectra were recorded at 150 MHz respectively. Mass spectra (MS) were measured with an Exactive Plus Orbitrap mass spectrometer via an ESI interface. Characterization of MR properties were measured at a Pharmscan 70/16 US (Bruker, Switzerland) magnetic resonance imaging scanner fitted with RF RES 300 1H 089/072 QSN TR AD volume coil.

Compound **3**

In a round bottom flask, 4-nitrobenzyl alcohol (1.0 g, 6.53 mmol) and DMAP were mixed with 25 mL of toluene. A solution of Bromoacetyl bromide (1.45 g, 7.18 mmol, 1.1 eq.) in 10 mL of toluene was slowly dropped and the reaction was stirred at room temperature for 5 h under N_2 atmosphere. The reaction was monitored by TLC (PE: EA = 5:1, Rf = 0.44). After the reaction was complete, the mixture was washed by saline and water. The organic phase were then dried over Na_2SO_4, filtered and evaporated in vacuum to afford product **3** as a light yellow solid (1.646 g, yield 92%). mp 44–46 °C. ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.25 - 8.24 (d, *J* = 8.4 Hz, 2H, -Ar), 7.55 - 7.54 (d, *J* = 9 Hz, 2H, -Ar), 5.30 (s, 2H, -OCH_2-), 3.91 (s, 2H, -CH_2-). ^{13}C NMR (150 MHz, Chloroform-*d*) δ 167.0, 148.1, 142.2, 128.6, 124.1, 66.4, 25.4.

Compound **4**

To a suspension of cyclen (1.0 g, 5.80 mmol) and sodium acetate (1.571 g, 19.16 mmol, 3.3 eq.) in DMA (15 mL) at –20°C was added a solution of t-butyl bromoacetate (3.623 g, 18.58 mmol, 3.2 eq.) in DMA (5 mL) dropwise over a period of 1 hour. The temperature was maintained at -20°C during the addition, after which the reaction mixture was allowed to come to room temperature. After 24 hours of vigorous stirring, the reaction mixture was poured into water (60 mL) to give a clear solution. Solid $NaHCO_3$ (2.52 g, 30 mmol) was added portion wise, and compound **4** precipitated as a white solid. The precipitate was collected by filtration and dissolved in DCM. The solution was washed with water, dried (Na_2SO_4), filtered and concentrated to about 5-10 mL. The crude product was purified by chromatography over silica gel with DCM:MeOH (30:1-15:1) to afford product. Yield: 1.86 g (62%). mp 179–181 °C. ^{1}H NMR (600 MHz, Chloroform-*d*) δ 3.36 (s, 4H, -CH_2-), 3.28 (s, 2H, -CH_2-), 3.09-2.86 (m, 16H, -CH_2-), 1.46-1.44 (m, 27H, -CH_3). ^{13}C NMR (150 MHz, Chloroform-*d*) δ 170.6, 169.7, 81.9, 81.8, 58.3, 51.4, 49.3, 47.6, 28.3, 28.3. HRMS (ESI^{+}): Calcd. for $C_{26}H_{51}O_6N_4$ $[M+H]^{+}$, 515.3803, Found, 515.3796.

Compound **5**

To a suspension of compound **4** (1.0 g, 1.94 mmol) in acetonitrile, K_2CO_3 powder (0.322 g, 2.33 mmol, 1.2 eq.) and subsequently compound **3** (0.586 g, 2.14 mmol, 1.1 eq.) in DCM was slowly added within 10 min.

Reaction was stirred under argon at room temperature overnight. The reaction was monitored by TLC (DCM:MeOH = 15:1, Rf = 0.45). The precipitated solids were removed by filtration and the filtrate was concentrated to give the crude product, which was purified by silica gel column chromatography using DCM:MeOH (30:1→20:1) to afford compound **5** as white solid. Yield: 1.225 g, 89%. mp 63-65 °C. ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.21 (dd, *J* = 16.4, 8.6 Hz, 2H, -Ar), 7.53 (dd, *J* = 16.4, 8.6 Hz, 2H, -Ar), 5.23 (s, 2H, -OCH_2-), 3.90-3.00 (m, 24H, -CH_2-), 1.46-1.44 (m, 27H, -CH_3). ^{13}C NMR (150 MHz, Chloroform-*d*) δ 172.9, 172.4, 172.2, 169.3, 147.9, 142.9, 128.6, 128.6, 124.0, 123.9, 82.5, 82.1, 65.4, 56.8, 56.3, 56.2, 55.9, 51.8, 51.4, 51.1, 50.7, 28.2. HRMS (ESI$^+$): Calcd. for $C_{35}H_{58}O_{10}N_5$ [M+H]$^+$, 708.4178, Found, 708.4178.

Compound **6** (DOTA-PNB)

For removal of the *t*Bu-group, compound **5** (0.775 g, 1.09 mmol) was dissolved in 20 mL 98%/1%/1% TFA/trimethylsilane/H_2O and the reaction mixture was stirred under argon at ice bath overnight. The reaction was monitored by LC–MS. After the reaction was complete, the solvent was concentrated to give the crude product, which was purified by RP-HPLC via reversed phase column chromatography (C18 column, 2%-90% MeCN : H_2O) to afford compound **6** as white solid. Yield: 371 mg (63%). mp 148–150 °C. ^{1}H NMR (600 MHz, D_2O) δ 8.25-8.24 (d, *J* = 9 Hz, 2H, -Ar), 7.64-7.63 (d, *J* = 7.8 Hz, 2H, -Ar), 5.32 (s, 2H, -OCH_2-), 3.87-3.79 (q, 4H, -CH_2-), 3.76 (s, 2H, -CH_2-), 3.61 (s, 2H, -CH_2-), 3.49-3.44 (m, 8H, -CH_2-), 3.12-3.01 (m, 8H, -CH_2-). ^{13}C NMR (150 MHz, D_2O) δ 177.9, 174.9, 173.0, 150.3, 146.1, 131.8, 126.7, 68.7, 59.5, 56.4, 55.8, 54.3, 54.2, 50.8. HRMS (ESI$^+$): Calcd. for $C_{23}H_{34}O_{10}N_5$ [M+H]$^+$, 540.2300, Found, 540.2294.

Probe **1** (Gd-DOTA-PNB)

Compound **6** (100 mg, 0.185 mmol) was dissolved in 10 ml deionized H_2O and the pH adjusted to 6.5-7.0 by the addition of NaOH (0.1 M). $GdCl_3 \cdot 6H_2O$ (75.8 mg, 0.204 mmol, 1.1 eq.) was slowly added as a solution in H_2O (0.2 mL) and the reaction was stirred at r.t. The pH of the solution was periodically checked and maintained to 6.5-7.0 with the addition of NaOH (0.1 M). The reaction was stirred until the pH was constant for 1 hour (4 hour total reaction time). Upon completion, the solvent was concentrated to give the crude product, which was purified by RP-HPLC via reversed phase column chromatography (C18 column, 2%–90% MeCN: H_2O). The product was lyophilized to probe **1** as a white fluffy solid. Yield: 78 mg (61%). mp 212–214 °C. HRMS (ESI$^+$): Calcd. for $C_{23}H_{31}O_{10}N_5Gd$ [M+H]$^+$, 695.1306, Found, 695.1299.

Compound **2** (Gd-DOTA)

DOTA (100 mg, 0.247 mmol) was dissolved in 10 mL deionized H_2O and the pH adjusted to 6.5–7.0 by the addition of NaOH (0.1 mol/L). $GdCl_3 \cdot 6H_2O$ (110.3 mg, 0.297 mmol, 1.2 eq.) was slowly added as a

solution in H_2O (0.3 mL) and the reaction was stirred at r.t. The pH of the solution was periodically checked and maintained to 6.5–7.0 with the addition of NaOH (0.1 mol/L). The reaction was stirred until the pH was constant for 1 hour (4 hour total reaction time). Upon completion, the solution was then adjusted to pH 9–10 by the addition of NaOH and the reaction was stirred for 20 minutes more, then filtered through a 0.45 µmol/L syringe filter. The solvent was concentrated to give the crude product, which was purified by RP-HPLC via reversed phase column chromatography (C18 column, 1%–5% MeCN: H_2O). The product was lyophilized to afford compound **2** (Gd-DOTA) as a white fluffy solid (18 mg, yield 13%). HRMS (ESI^+): Calcd. for $C_{16}H_{26}O_8N_4Gd$ $[M+H]^+$, 559.0908, Found, 559.0942.

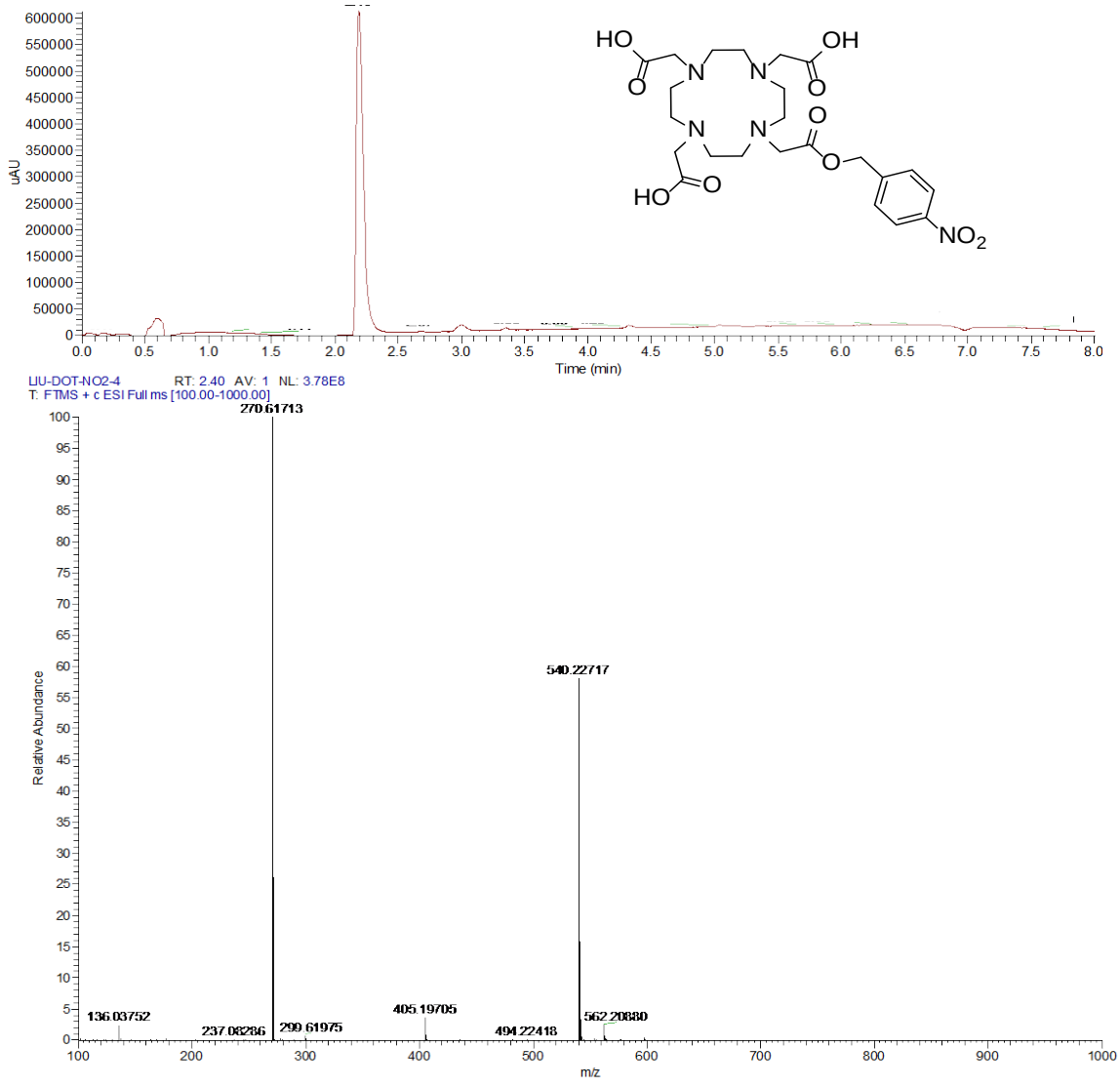


Figure S1 HPLC trace (top) and ESI-MS (bottom) of the ligand, DOTA-PNB (**6**)

2. Time response of probe 1 to nitroreductase monitored by LC–MS.

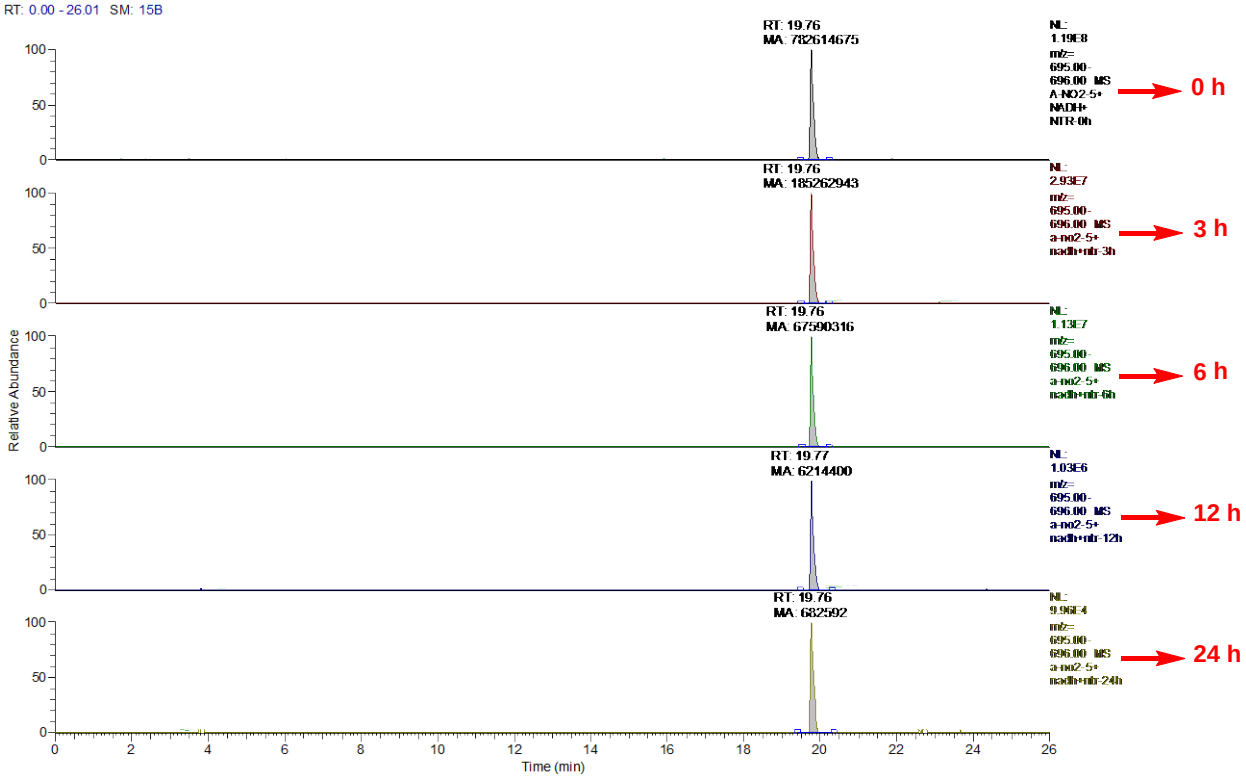


Figure S2 Time-dependent ESI profile of probe **1** (200 µmol/L) cleaved with nitroreductase (30 µg/mL) monitored by analytical LC–MS equipped with an Electrospray Ionization mass spectra (ESI) and using a Kromasil C18 Column. Spectra clearly show the conversion of **1** over time after incubation with nitroreductase. Kinetics were plotted in **Fig. 2** based on calculating the area under the curve of **1**.

3. ESI-MS proof for the formation of the final product

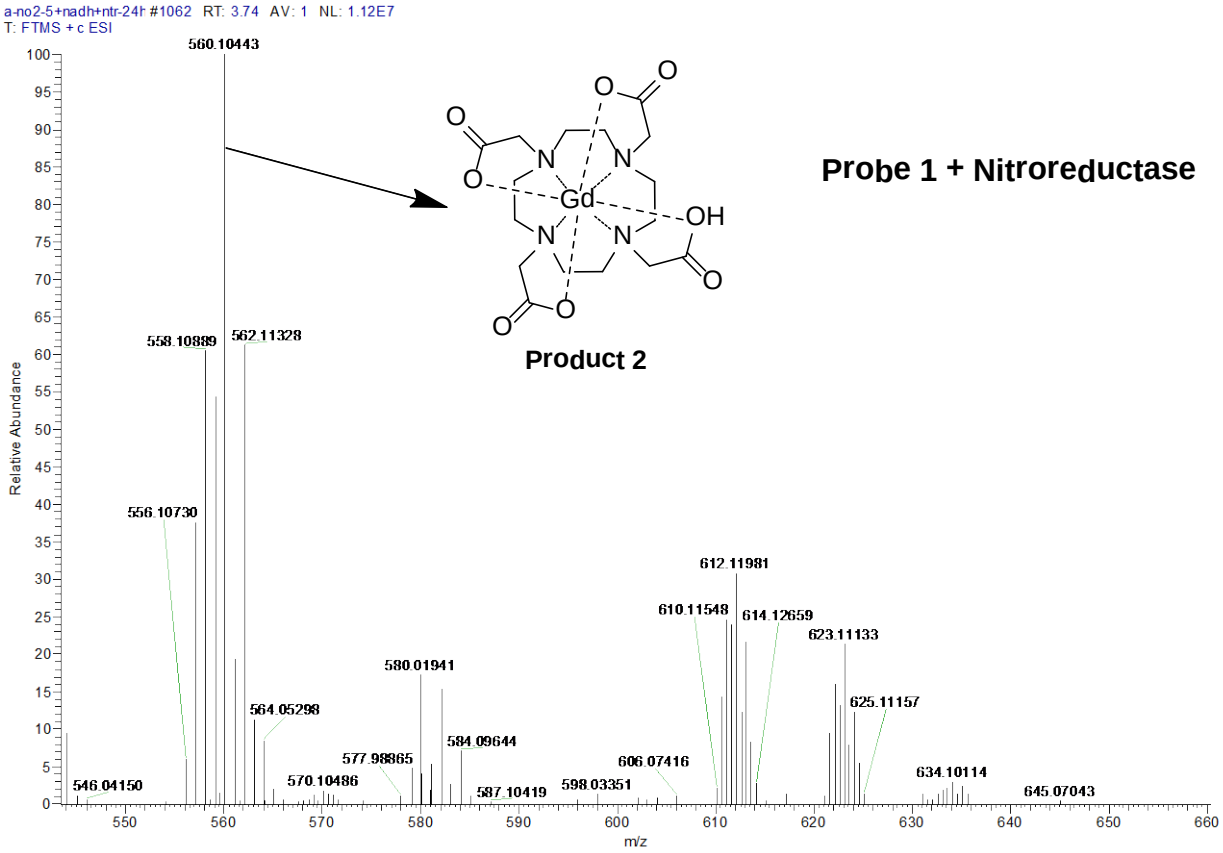


Figure S3 ESI-MS spectrum of the reaction solution of probe **1** (200 μmol/L) with nitroreductase (30 μg/mL).

4. HPLC analysis for the Nitroreductase reaction system.

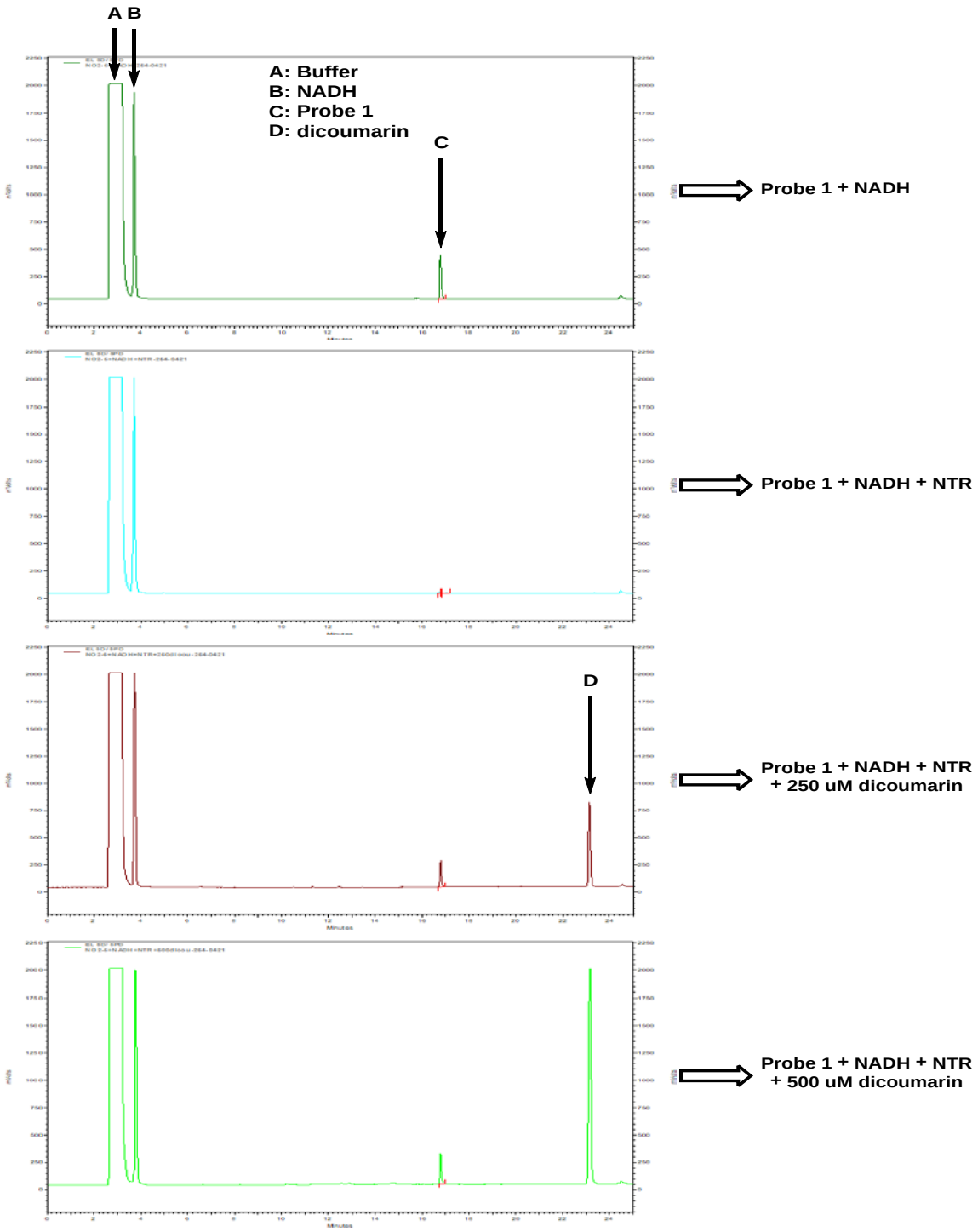


Figure S4 HPLC trace of probe **1** (200 µmol/L) cleaved with nitroreductase (30 µg/mL) monitored by analytical HPLC equipped with an Evaporative Light Scattering Detector (ELSD) using an Kromasil C18 Column. Spectra clearly show the conversion of **1** after incubation with nitroreductase and the persisting

concentrationg of **1** due to co-incubation with the NTR-inhibitor dicoumarin. The percentage of probe **1** in **Fig. 3** based on calculating the area under the curve of **1**.

5. ESI-MS spectra of the reaction of probe 1 with *Escherichia coli*.

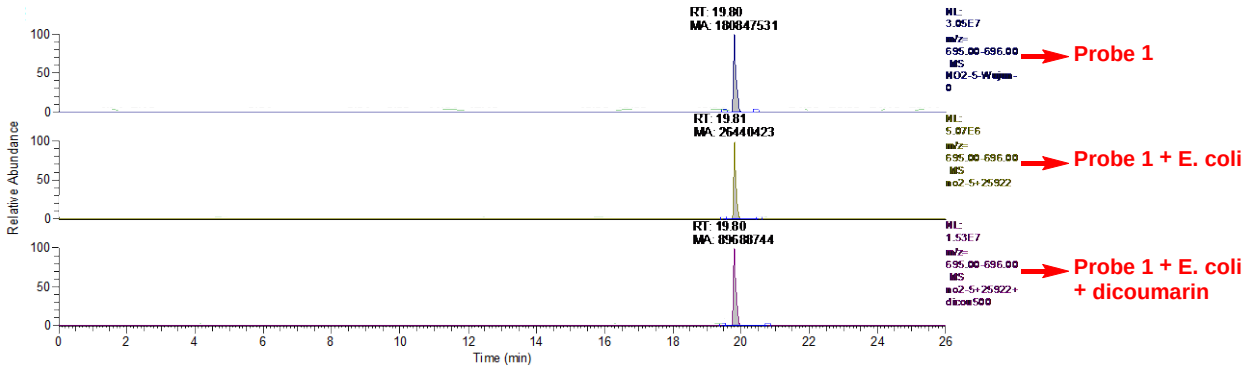
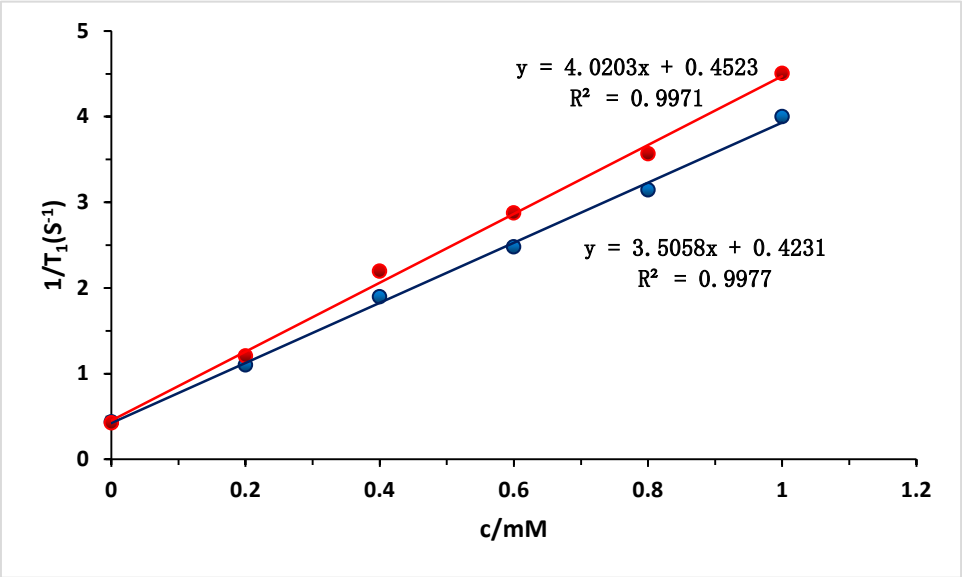


Figure S5 ESI trace of probe **1** (200 µmol/L) incubated with E. coli (OD = 6) and dicoumarin (500 µmol/L) monitored by analytical LC–MS equipped with an Electrospray Ionization mass spectra (ESI) and using a Kromasil C18 Column. Spectra clearly show the significant weaker conversion of **1** after incubation with *E. coli* in the presence of NTR inhibitor dicoumarin. The percentage of probe **1** is based on calculating the area under the curve of **1**.

6. Relaxivity measurement of compound 1 and 2

a



b

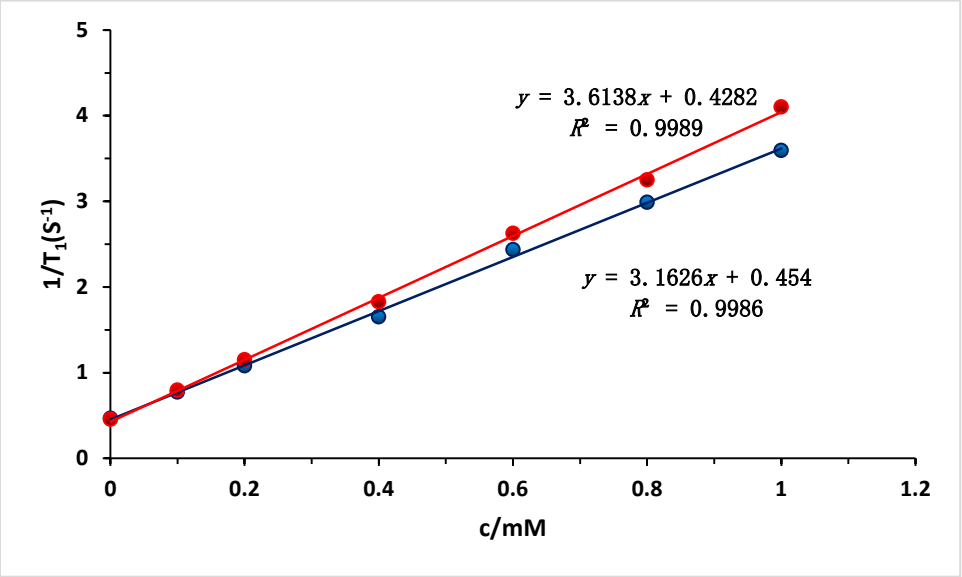


Figure S6 High field (7 T, 25 °C) relaxivity of compound **1** (blue) and **2** (red) in 10 mmol/L PBS (a) and 0.9% NaCl (b). TE = 8 ms, TR = 5500, 3000, 1500, 800, 400, and 200 ms, FOV = 50 × 50 mm^2, MTX = 256 × 192, number of axial slices = 1, slice thickness = 1.0 mm, and averages = 1 using a T_1MapRARE pulse sequence.

7. T_1 measurements of probe 1 to different concentrations of NTR

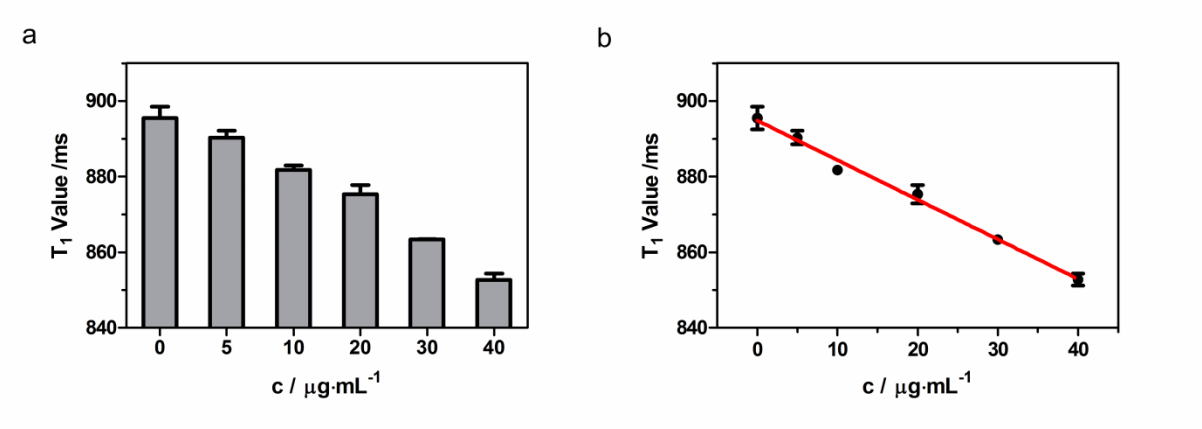


Figure S7 (a) T_1 values of probe **1** to different concentrations of NTR (0, 5, 10, 20, 30 and 40 μg/mL), 500 μmol/L NADH as a coenzyme. (b) A linear correlation between T_1 values and concentrations of NTR. Data represent mean values ± standard deviation, n = 2.

8. *In vitro* MR imaging of Hela cells treated with probe 1

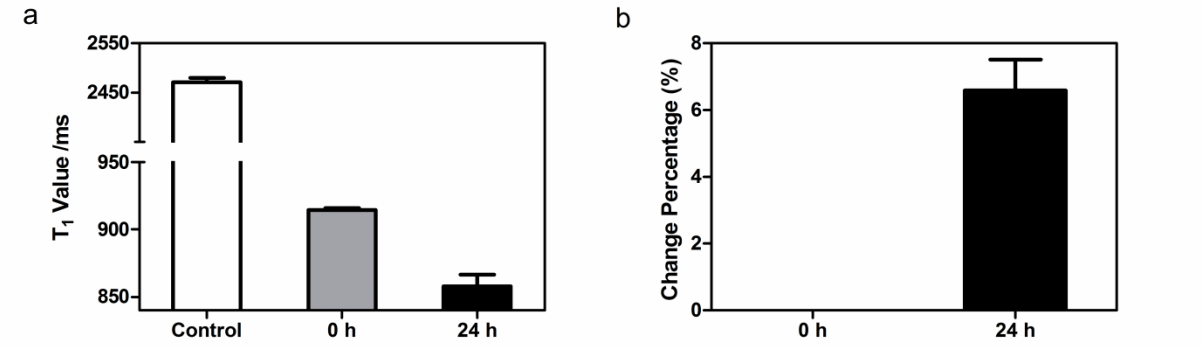


Figure S8 Cellular MR studies of incubating with probe **1**. (a) T_1 values (7 T) of Hela cells after incubation with 200 μmol/L of probe **1** for 0 h or 24 h. (b) Change percentage in R_1 ($1/T_1$) of probe **1** in Hela cells. T_1 value was measured with a Pharmscan 70/16 US (Bruker, Switzerland) imaging scanner at r.t., using the standard inversion recovery program. Data represent mean values ± standard deviation, n = 3.

9. Cell Viability Assays

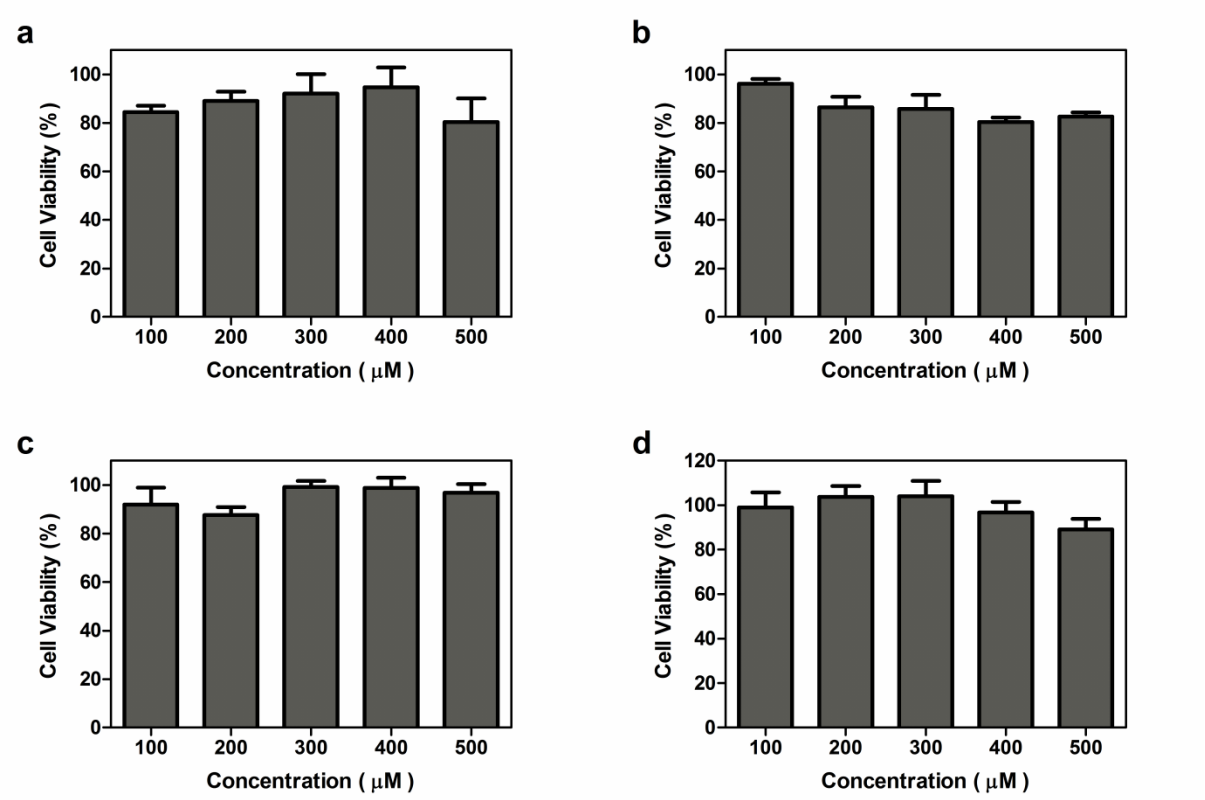
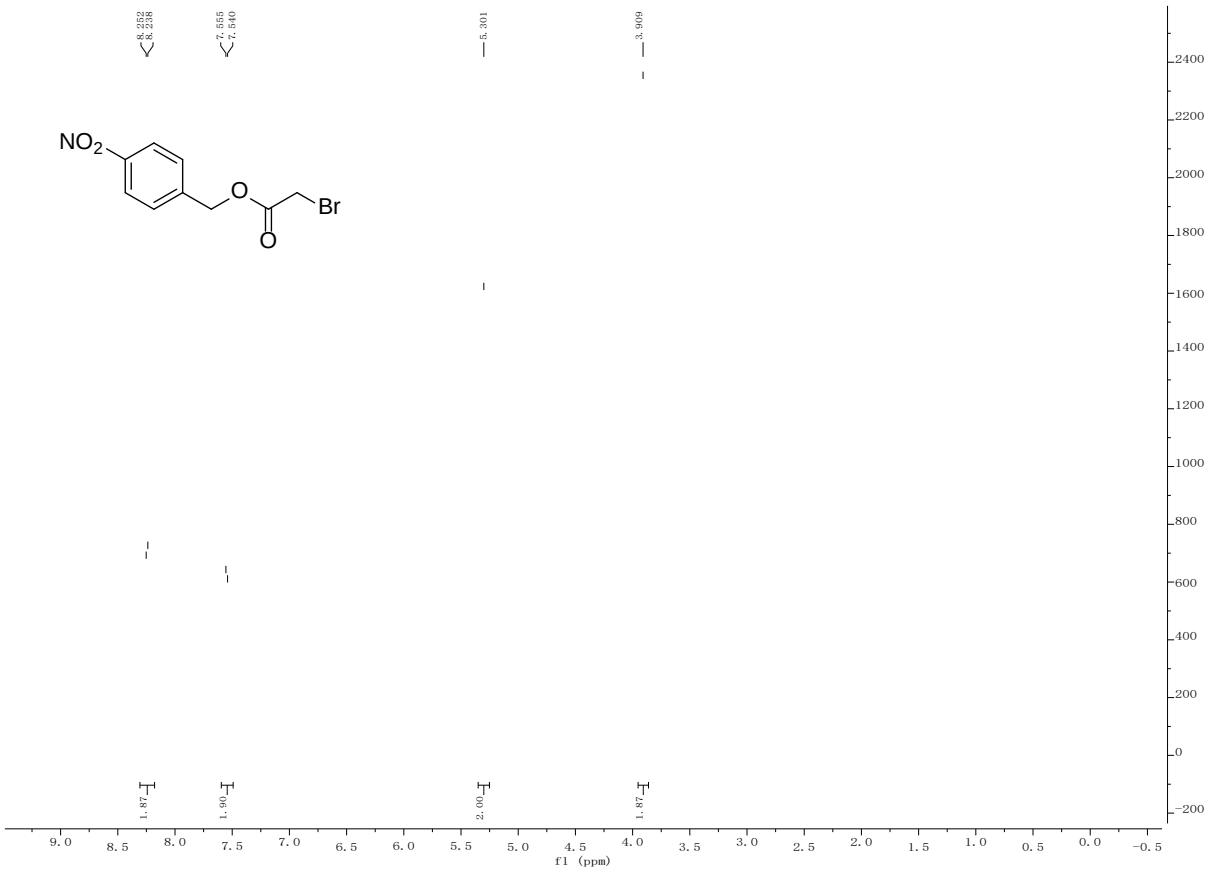
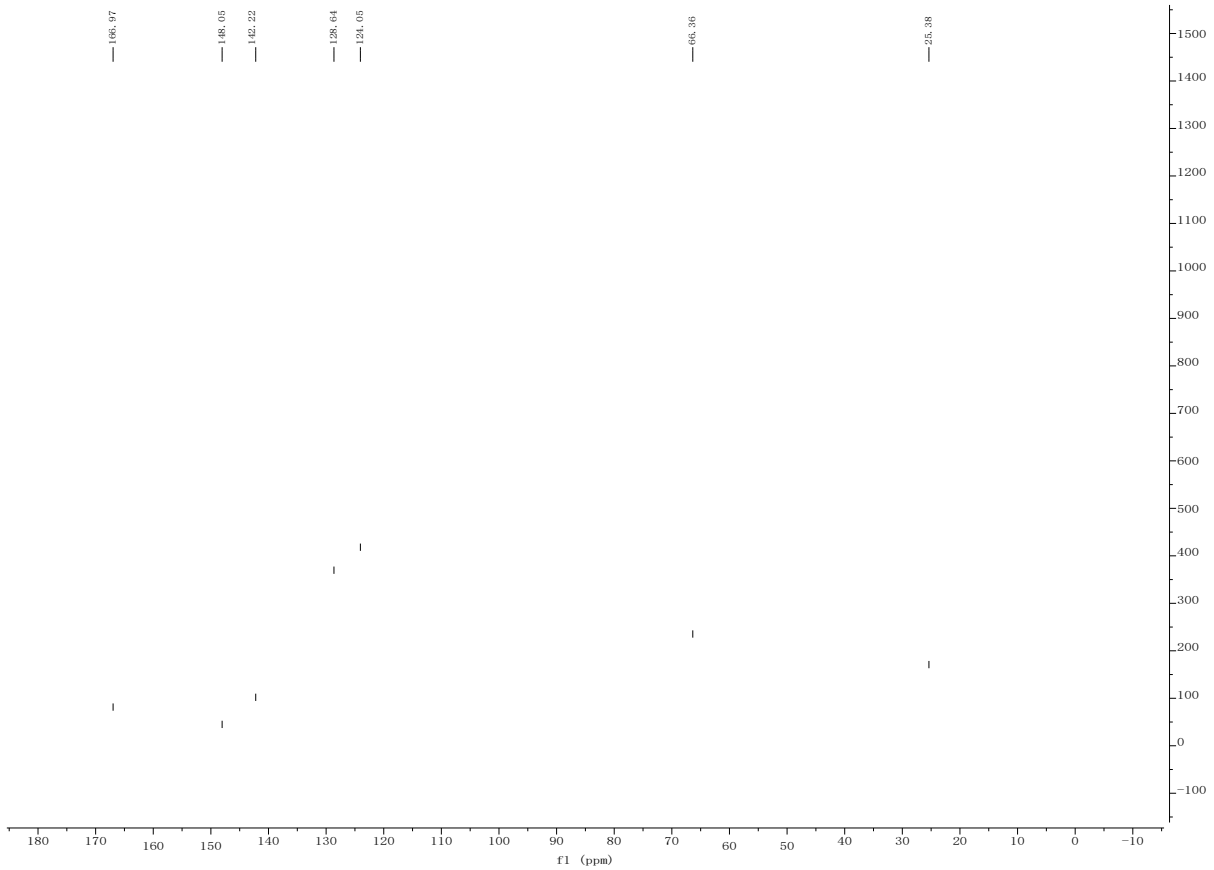


Figure S9 The cell viability of RAW (a), 293A (b), L6 (c), HepG2 (d) cells after 48 hours incubation with various concentrations (100, 200, 300, 400, 500 μmol/L) of probe **1**. The viability of the cells without probe **1** is defined as 100%. Results represent the mean of results from 3 separate wells. Error bars show the standard error of the mean.

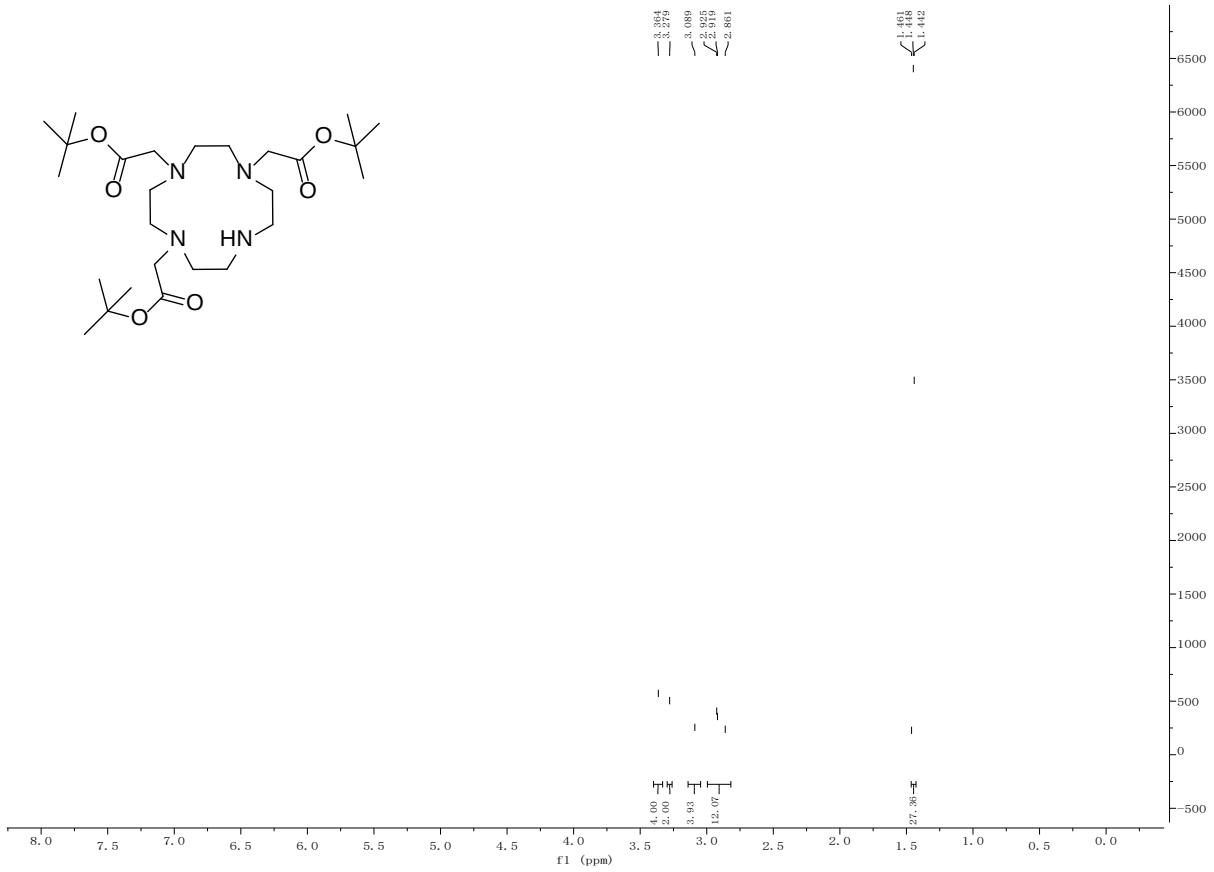
10. ^{1}H NMR, ^{13}C NMR and ESI mass spectra of compound 1–6.



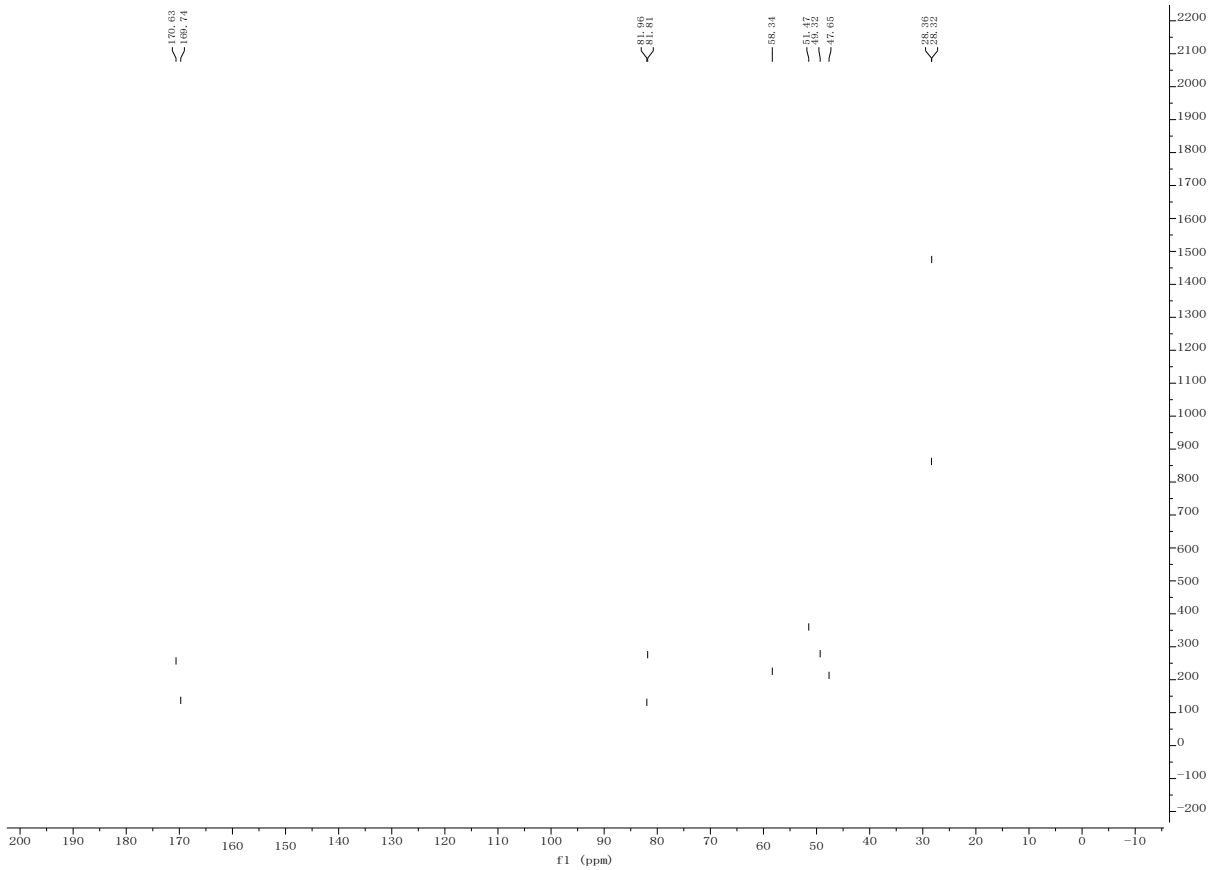
^{1}H NMR (600 MHz, Chloroform-*d*) of compound **3**



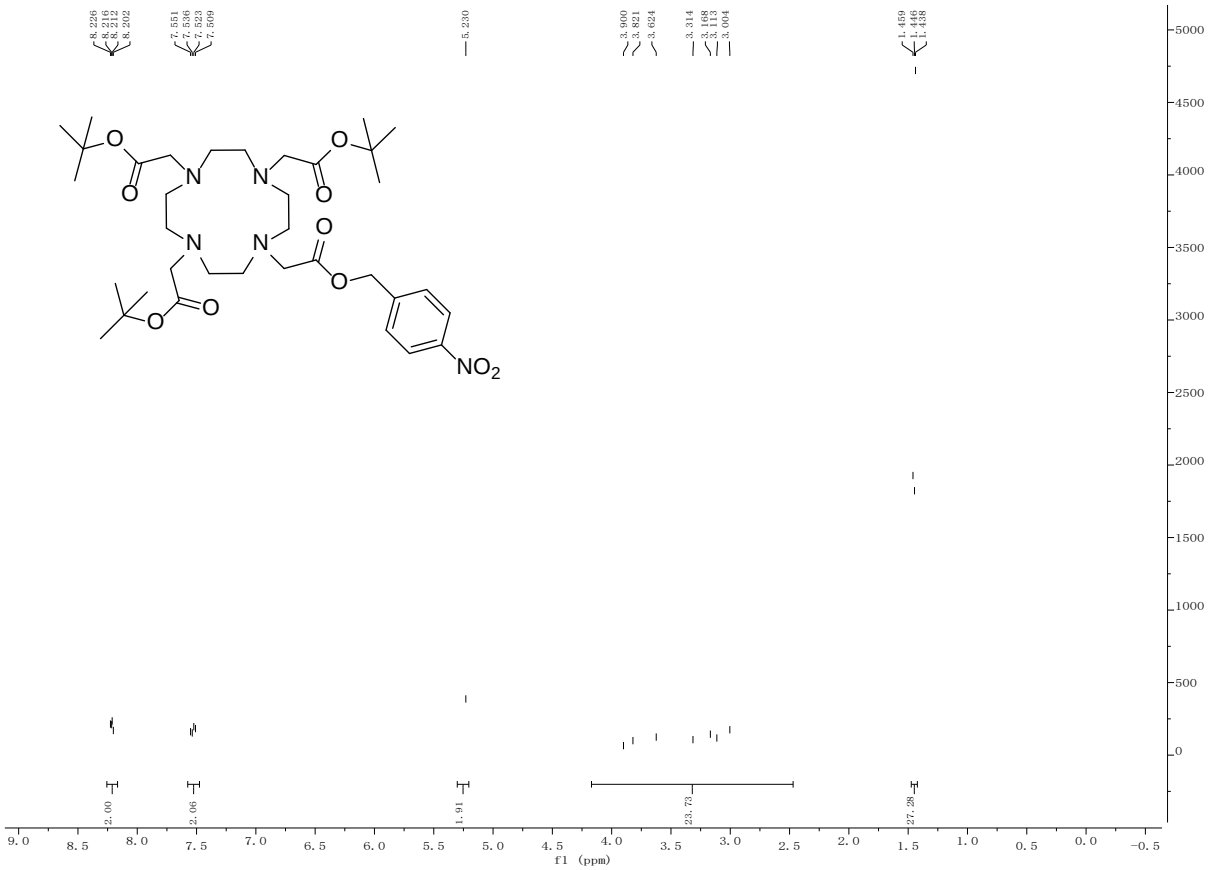
^{13}C NMR (150 MHz, Chloroform-*d*) of compound **3**



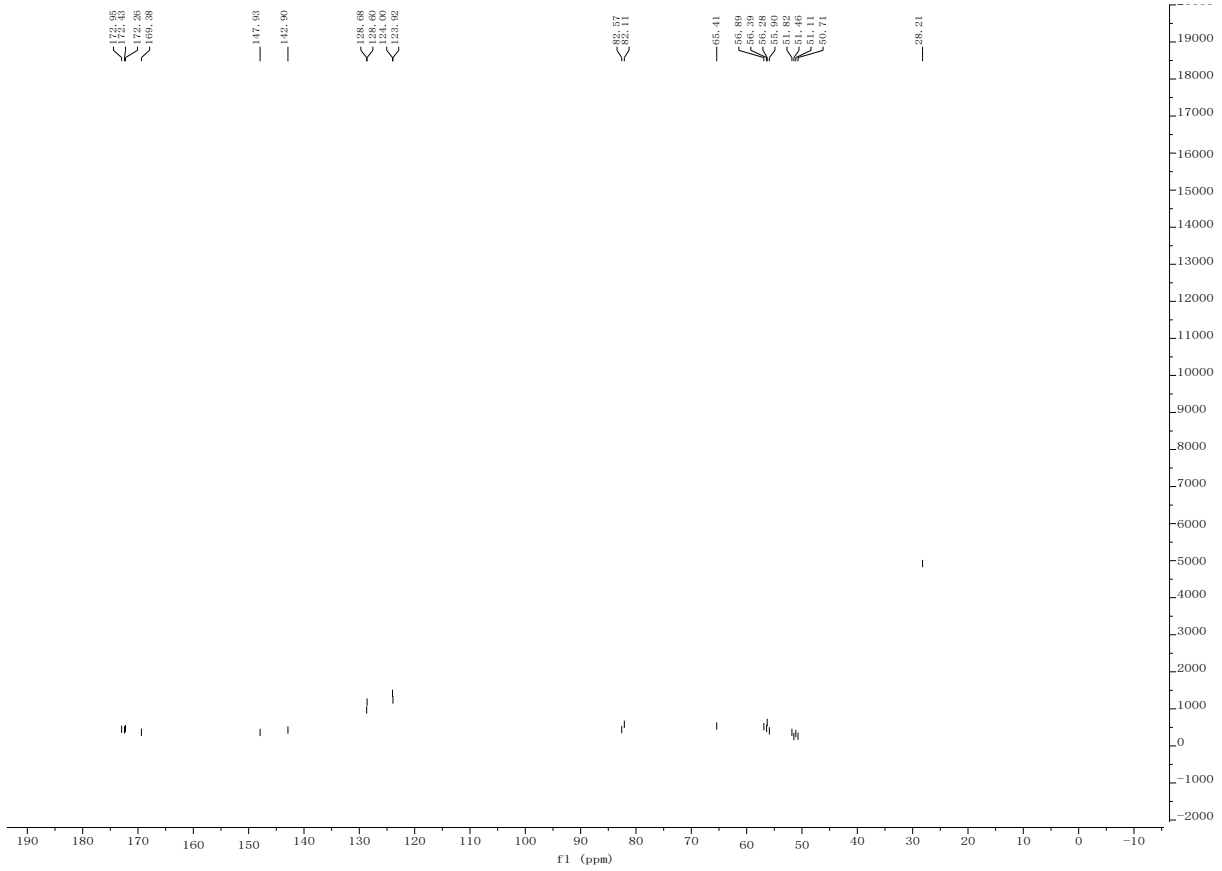
^{1}H NMR (600 MHz, Chloroform-*d*) of compound **4**



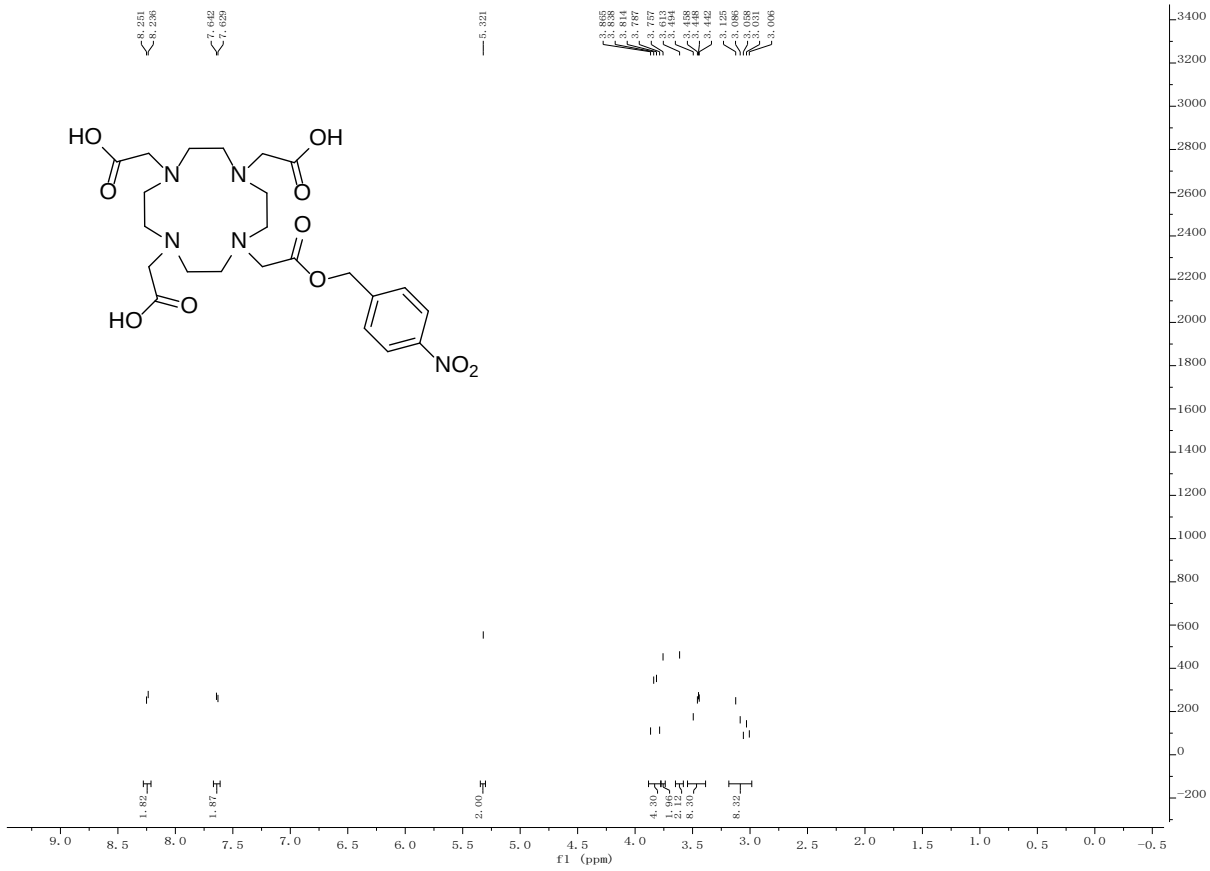
^{13}C NMR (150 MHz, Chloroform-*d*) of compound **4**



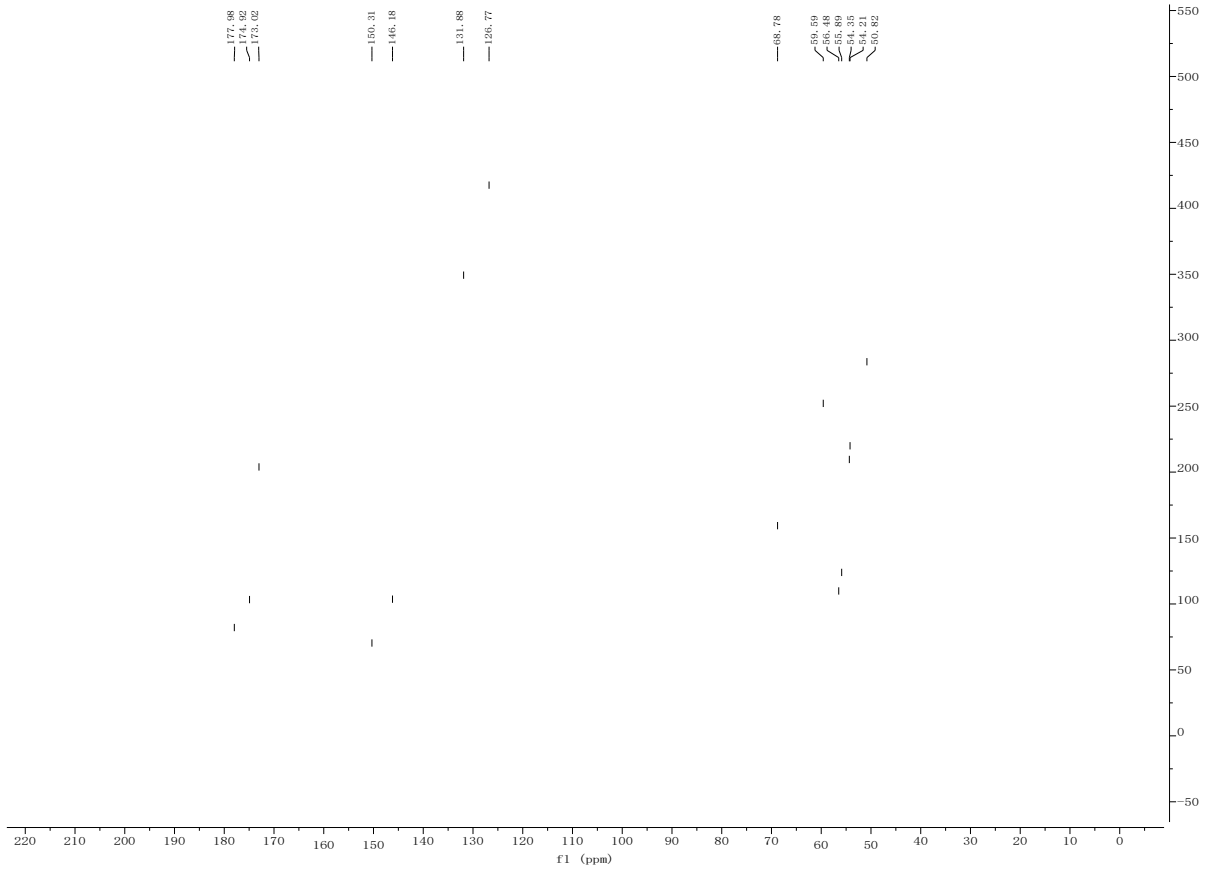
^{1}H NMR (600 MHz, Chloroform-*d*) of compound **5**



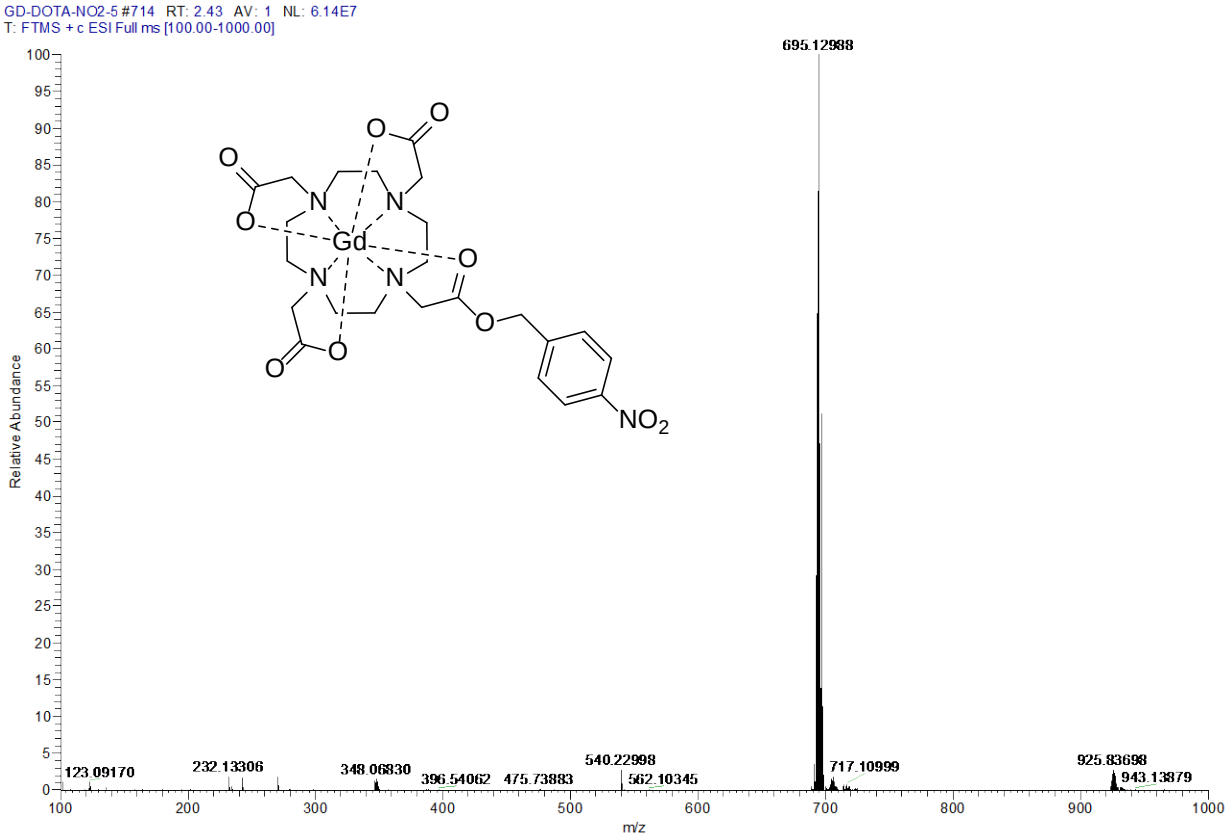
^{13}C NMR (150 MHz, Chloroform-*d*) of compound **5**



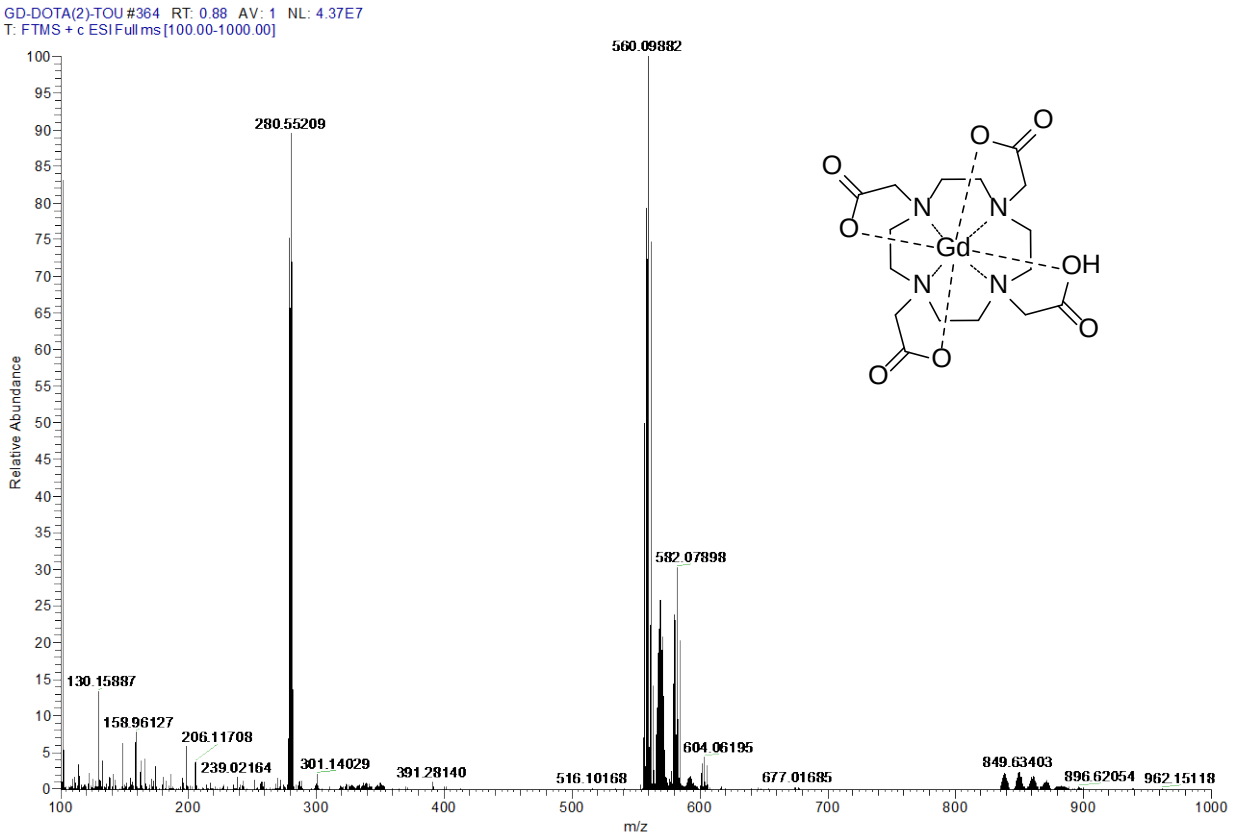
^{1}H NMR (600 MHz, D_2O) of compound **6**



^{13}C NMR (150 MHz, D_2O) of compound **6**



ESI-MS spectra of probe **1 (Gd-DOTA-PNB)**



ESI-MS spectra of compound **2 (Gd-DOTA)**