Supporting Information

Nanobody Site-Specific C-Terminal Dinitrophenylation for mimicking the antibody Fc Biological Functions

Haofei Hong,[a] Zhifang Zhou,[a] Kun Zhou,[a] Shaozhong Liu,[a] Zhongwu Guo,[*b] and Zhimeng Wu[*a]

[a.] Key Laboratory of Carbohydrate Chemistry & Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi, 214122, China; E-mail: zwu@jiangnan.edu.cn

[b.] Department of Chemistry, University of Florida, 214 Leigh Hall, Gainesville, Florida 32611, United States of America; E-mail: zguo@chem.ufl.edu

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1. Supporting Figures

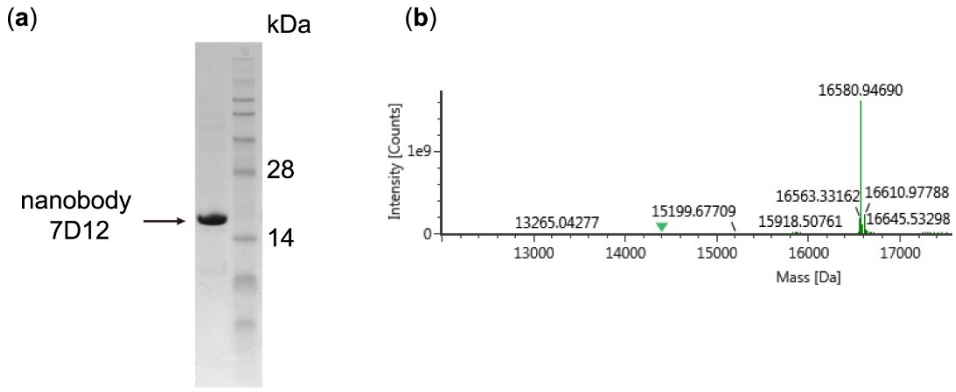

Figure S1. SDS-PAGE and MS analysis of purified nanobody 7D12. (**a**), SDS-PAGE of nanobody 7D12; (**b**) MS of naobody 7D12, observed: 16580.9 Da; calculated MW: 16581.1 Da.

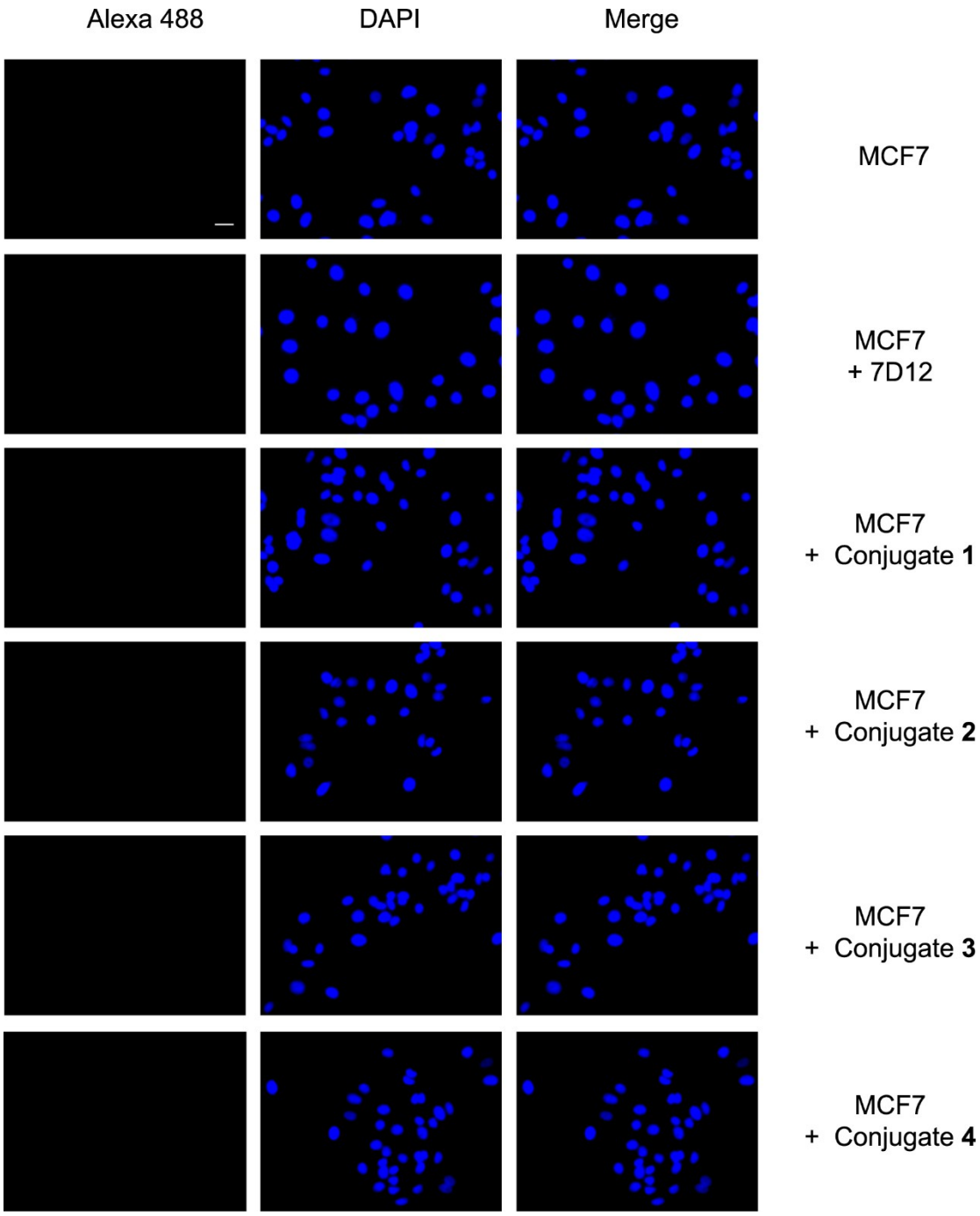

Figure S2. Immunofluorescence of MCF7 cells treated with nanobody 7D12, conjugate **1**-4.

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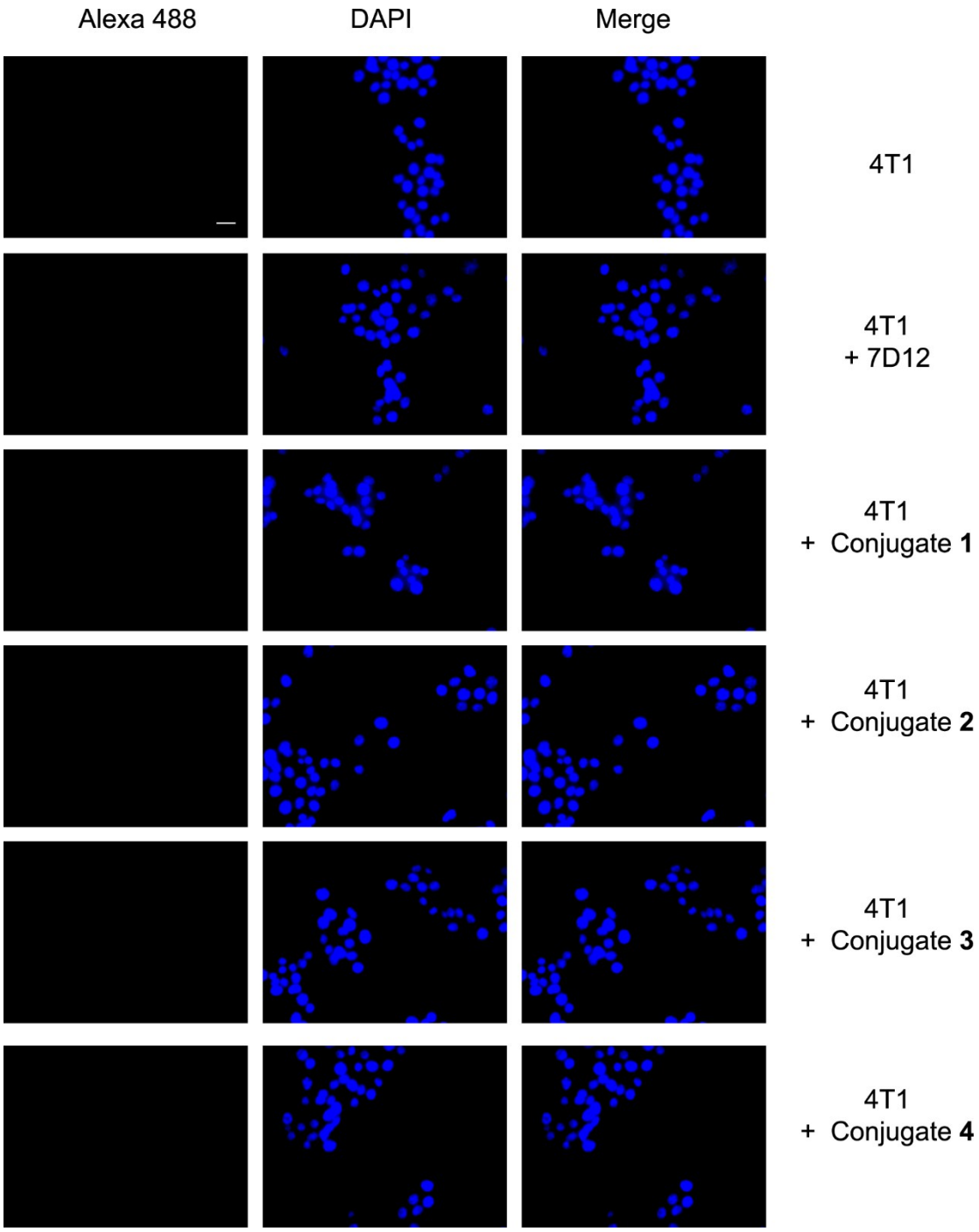


Figure S3. Immunofluorescence of 4T1 cells treated with nanobody 7D12, conjugate **1** -4.

Scar Bar: 20 μm.

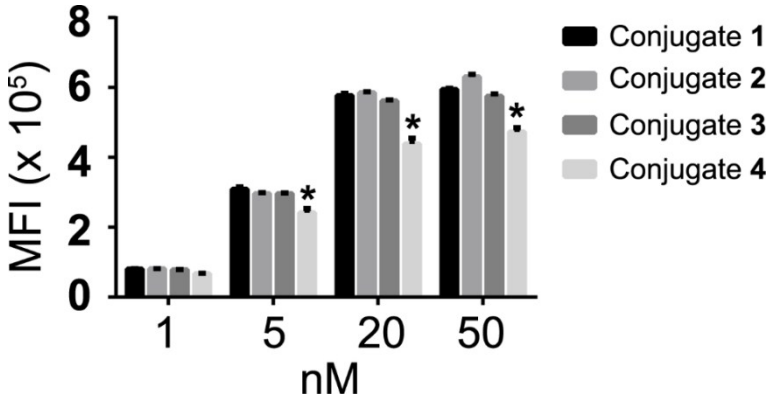


Figure S4. Flow cytometry analysis of A431 cells treated with different concentrations of 7D12-DNP conjugate 1-4. *: $p<0.05$.

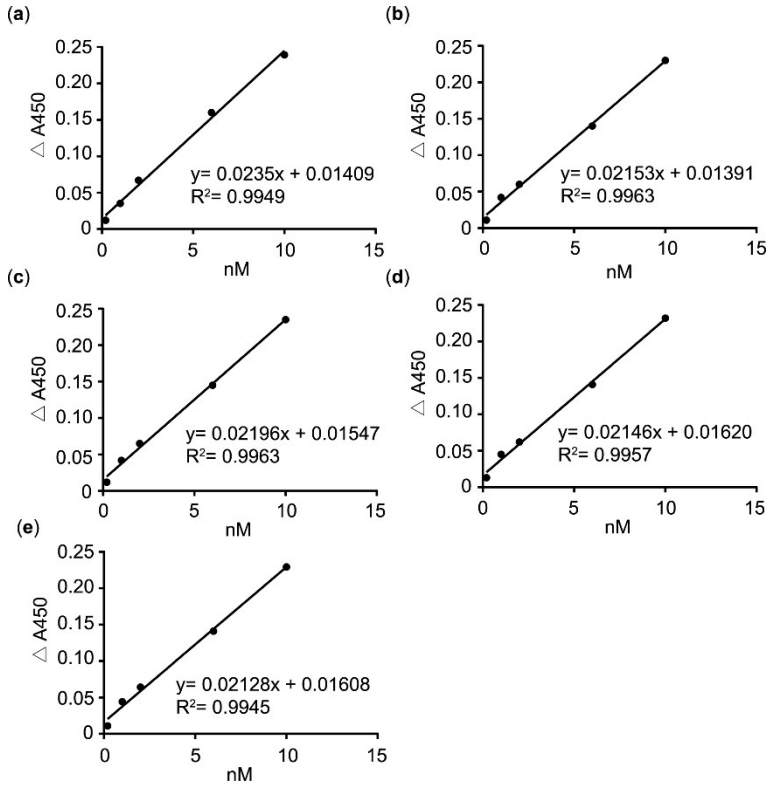


Figure S5. The standard curves for nanobody concentration determination. The standard curve of nanobody 7D12 (**a**), conjugates **1** (**b**), conjugate **2** (**c**), conjugate **3** (**d**) and conjugate **4** (**e**) were generated by ELISA using human recombinant EGFR as the antigen. $\Delta A450 = A_x - A_0$, where A_x is the OD_{450} value of samples containing x nM of nanobodies, A_0 is the OD_{450} value of samples containing 0 nM of nanobodies.

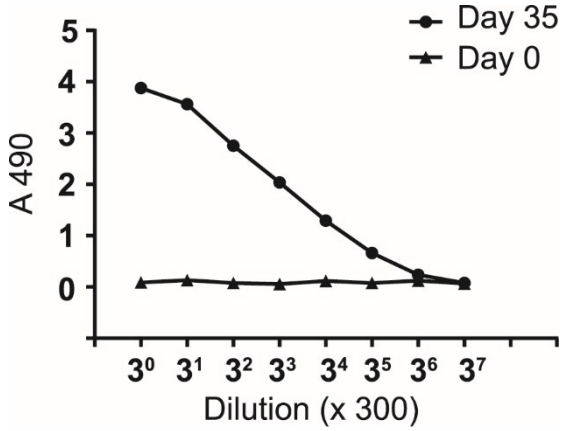


Figure S6. The titer of anti-DNP antibodies in Balb/c mice at day 0 and day 35. The calculated titer at day 35 was more than 10^5.

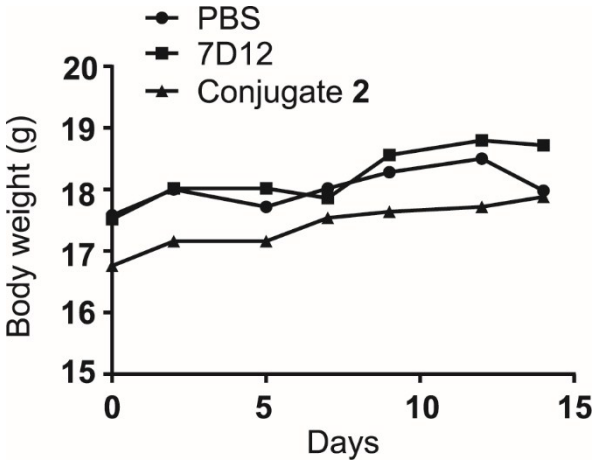


Figure S7. The body weight of SCID mice treated with PBS, nanobody 7D12 or conjugate **2** in the presence of anti-DNP mouse serum.

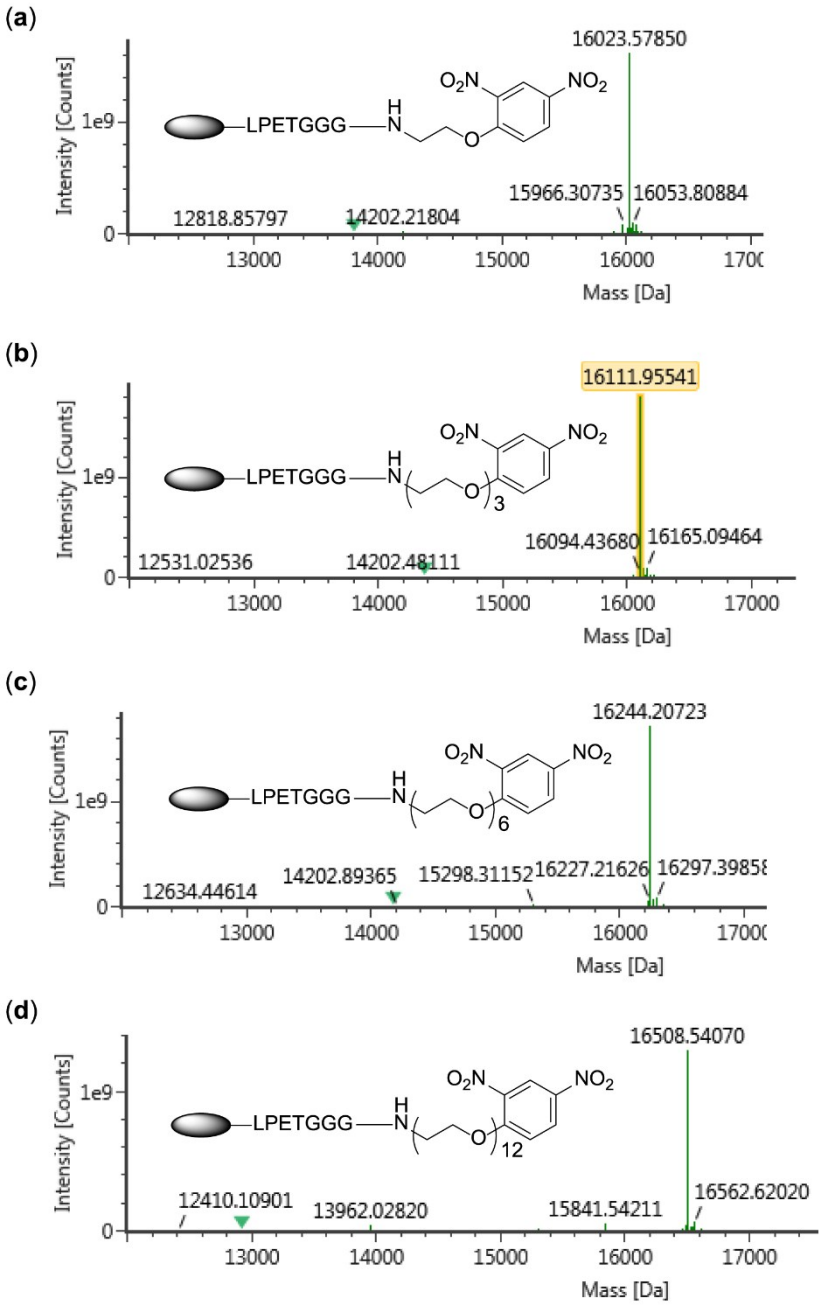


Figure S8. MS of conjugates **1**, **2**, **3** and **4**. (**a**) MS of conjugate **1**, observed: 16023.5 Da; calculated MW: 16024.3 Da. (**b**) MS of conjugate **2**, observed: 16111.9 Da; calculated MW: 16112.3 Da. (**c**) MS of conjugate **3**, observed: 16244.2 Da; calculated MW: 16244.4 Da. (**d**) MS of conjugate **4**, observed: 16508.5 Da; calculated MW: 16508.2 Da.

2. General Information

Materials. The pET28a expression vector and *E.coli* BL21 (DE3) were purchased from Novagen and American Type Culture Collection (ATCC, USA), respectively. Gene and primers were synthesized in General Biosystems Co. Ltd. Phenol-free RPMI 1640 (Cat. No. 11835-030), 12% polyacrylamide gel (Cat. No. NP0032BOX), Rabbit anti-DNP IgG antibody (Cat. No. A-6430) and Alexa Fluor 488 conjugated Rabbit anti-DNP IgG antibody (Cat. No. A-11097) were purchased from Thermo Fisher Scientific. Dulbecco's Modified Eagle Medium (DMEM, Cat. No. SH30022.01), Roswell Park Memorial Institute medium (RPMI medium, Cat. No. SH30809.01), Fetal Bovine Serum (FBS, Cat. No. SH30084.03), Penicillin-Streptomycin solution (Cat. No. SV30010) and HiPrep Desalting column were all from GE Healthcare. The recombinant human EGFR (Cat. NO. Z03194) and HRP-conjugated Rabbit anti-myc tag IgG antibody (Cat. No. A00173) were from Genscript. Rabbit complement was from Sigma- Aldrich. The BCA kit, TMB kit, LDH cytotoxicity assay kit and CCK8 assay kit were purchased from Beyotime. Ni-IDA magnetic beads were from Beaver and Ni-NTA agarose was from Yeasen. All the secondary antibodies and other reagents were purchased commercially.

Cell culture. Three cell lines (gifts from Prof. Guan Feng, Northwest University, China) were used. Human epidermoid carcinoma A431 and human breast cancer MCF7 were grown in DMEM supplemented with 10% FBS, 1% penicillin- streptomycin. Murine breast cancer 4T1 was grown in RPMI supplemented with 10% FBS, 1% penicillin- streptomycin. All cells were cultured at 37°C in a 5% CO_2 humidified atmosphere.

SrtA expression. *E. coli* BL21(DE3) harboring pET28a-Δ59eSrtA gene was constructed and used to express SrtA following our previous protocol.[1] Briefly, the positive clone was propagated in Luria–Bertani (LB) medium containing 100 μg/mL of kanamycin at 37°C overnight with constant shaking at 200 rpm. The overnight culture was diluted in optimized medium (0.48 g/L glycerol, 1.37 g/L tryptone, 0.51 g/L yeast extract, MOPS 0.5 g/L, PBS 180 mL/L) and then shaken at 37°C and 200 rpm. When the OD600 value reached 0.6, *iso*-propyl β-D-thiogalactoside (IPTG) (final concentration: 1 mM) was added to induce target protein expression, and incubation was continued for another 8 h at 30°C. SrtA purification was performed using Ni-IDA magnetic beads (Beaver, Suzhou, China) according to the manufacturer's instructions.

Nanobody 7D12 expression. Synthetic 7D12 gene was used as for PCR with forward primer 5'-CATGCCATGGCACAGGTGAAACTGGAAGAAAGTG-3' (Nco I underlined) and reverse primer 5'-CGCGGATCCTCAGTGGTGGTGATGATGATG-3' (BamH I underlined). The PCR product was digested with Nco I/BamH I and then ligated into the similarly restriction-digested expression vector pET-28a. The recombinant plasmid was verified by DNA sequencing and named as pET28a-7D12. Thereafter, pET28a-7D12 was transformed into *E. coli* BL21(DE3). A single colony of *E. coli* BL21(DE3) cells harboring the recombinant plasmid was inoculated into LB medium containing 100 μg/mL of kanamycin and grown at 37°C and 200 rpm overnight. The seed culture (2%) was added into Terrific-Broth (TB) medium containing 100 μg/mL of kanamycin at 37°C until the OD600 value reached 0.6 and then incubated with 0.5 mM IPTG at 16°C for 24 h. After the fermentation was completed, cells were harvested by centrifugation at 8000 rpm for 5 min. After the supernatant was discarded, the cell pellet was resuspended in lyse buffer (Tris/HCl 10 mM, NaCl 500 mM, pH = 7.8) and disrupted on ice by sonication. After centrifugation at 9000 rpm for 20 min, the lysis was collected and then incubated with Ni–NTA agarose at 4 °C for 2 h in a gravity-flow column. Subsequently, the agarose was washed with a gradient of imidazole (0–40 mM) to remove other proteins. The *C*-terminal His_6-tagged 7D12 was eluted from the agarose with 500 mM imidazole solution and then desalted using a HiPrep Desalting column on ÄKTA avant. The concentrations of the target proteins were determined using the BCA kit.

SrtA-mediated ligation for nanobody 7D12-DNP conjugate synthesis. DNP derivatives **13**, **14**, **15** or **16** (500 μM), nanobody 7D12 (20 μM), and SrtA (5 μM) were mixed in a reaction buffer (Tris/HCl 50 mM, NaCl 150 mM, $CaCl_2$ 5 mM, pH = 7.5, 1.0 mL), the mixture was incubated at 16°C for 2-4 h with constant shaking at 200 rpm. Then, nickel-magnetic beads (500 μL) were added into the mixture to capture and remove SrtA and unreacted nanobody 7D12, which was vortexed at 4°C for 30 min. The supernatant containing the desired conjugates was collected and subjected to purification with a Sephadex G25 column using water as the eluent to provide 7D12-DNP conjugate **1**-**4** (yield: 1, 85%; 2, 85%; 3, 75%; 4, 75%). They were filtered through a sterile filter (0.22 μm) and stored at -20°C. The reaction yields were determined by protein analysis using the BCA kit. The final products were subjected to SDS-PAGE analysis to determine their purity and molecular weights.

Western blot. After nanobody 7D12 and 7D12-DNP conjugates were pretreated with sample buffer (No.

LC5925, Thermo) at 99°C for 10 min, they were run on a 12% polyacrylamide gel and then transferred onto a PVDF membrane. The membrane was blocked with 3% BSA in Tris-buffered saline and Tween 20 (TBST) at 37°C for 30 min. Subsequently, the membrane was incubated with rabbit anti-DNP IgG antibodies (1μg/mL) at 4°C overnight. Unbound antibodies were removed by washing three times with TBST. Then, the membrane was treated with HRP-conjugated goat anti-rabbit IgG (H+L) antibodies with a final concentration of 0.5 μg/mL (diluted in PBS containing 1% BSA). After incubation at 37°C for 60 min, the washing steps were repeated as described above. The membrane was finally imaged using a TMB kit on the Gel Doc XR System.

Immunofluorescence analysis. Cells were seeded on sterile coverslips in 24-well plates until 50-70% confluent is reached. Cells were incubated with 100 μL of 7D12-DNP conjugates or nanobody 7D12 (100 nM) or 100 μL of medium (negative control), respectively, in the presence of 20 μg/mL of Alexa 488 conjugated rabbit anti-DNP IgG antibodies at 37 °C for 30 min. Next, cells were washed 3 times with 500 μL of dulbecco's phosphate-buffered saline (DPBS) and fixed with 4% paraformaldehyde for 10 min, followed by another washing step. Cells were stained with 5 μg/mL of 4',6-diamidino-2-phenylindole (DAPI) for 5 min. Thereafter, cells were treated with a drop of Antifade Mounting Medium and observed with a fluorescence microscope.

Flow Cytometry. Cells were cultured and detached with trypsin-EDTA and then resuspended and diluted in flow cytometry buffer (2% BSA in DPBS) to 4.0×10^5 cells/mL. Forty thousand of these cells were added into tubes containing 100 μL of 7D12-DNP conjugates or nanobody 7D12 (50 nM), followed by addition of Alexa 488-conjugated rabbit anti-DNP IgG antibodies (20 μg/mL). In the negative control group, cells were treated with DPBS instead of nanobody. These cells were incubated on ice for 30 min, washed with flow cytometry buffer (2×500 μL) and then resuspended in 200 μL of flow cytometry buffer. Finally, they were analyzed using an Accuri C6 flow cytometer (BD Biosciences), and data analyses were performed with the FlowJo software.

Antibody-dependent cell-mediated cytotoxicity (ADCC) assay. A431 cells were seeded in 96-well plates at a concentration of 10,000 cells/well. After 12 h of incubation at 37°C, the cells were washed with ADCC buffer (phenol-free RPMI 1640 containing 1% penicillin-streptomycin and 5% FBS) and then treated with different concentrations of 7D12-DNP conjugates or nanobody 7D12 (0.2, 2, 20, 100 nM) in the presence

of rabbit anti-DNP IgG antibodies (20 μg/mL) at 37°C for 30 min. Excessive proteins were removed by washing (×3 times) with ADCC buffer. Next, freshly isolated human peripheral blood mononuclear cells (PBMC, 2× 10^6/mL, 100 μL) were added to each well at an effector-to-target (E/T) ratio of 20:1. Following the incubation period (37 °C, 5% CO_2 for 4 h), 60 μL of the supernatant was transferred onto a new black 96-well plate. Cytotoxicity was then assessed by measuring LDH release at 490 nm wavelength using the LDH kit. Spontaneous LDH release by effector cells were detected by the same method. The maximum killing was achieved by adding 10×lysis solution to target cells. Specific cytotoxicity was calculated by the following equation:

$$\%\ \text{ADCC} = \frac{\text{A (experimental)} - \text{A (spontaneous)}}{\text{A (maximum)}} \times 100$$

Where A(spontaneous) is the OD_{490} value of LDH spontaneously released by effector cells; A(experimental) is the OD_{490} value of LDH released by cells respectively treated with 7D12-DNP conjugates or nanobody 7D12 in the presence of anti-DNP IgG antibody and PBMC; A(maximum) is the OD_{490} value of cells completely lysed with 1% Triton X-100.

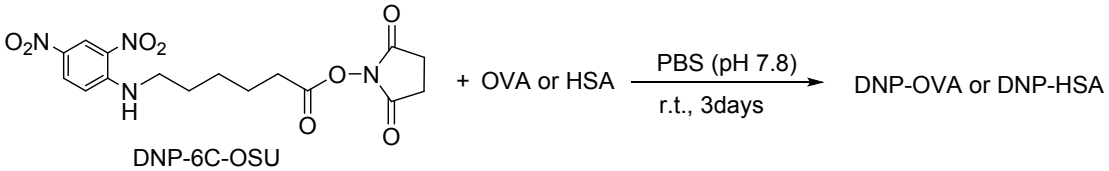
Complement-dependent cytotoxicity (CDC) assay. A431 cells were grown at 37°C overnight in 96-well plates (4000 cells/well). The old medium was removed by washing 2 times with DPBS. Then, 90 μL of CDC buffer (phenol-free RPMI 1640 containing 1% penicillin-streptomycin), 10 μL of 7D12-DNP conjugates or nanobody 7D12 (1.0 μM), and 1 μL of anti-DNP IgG antibodies (2 mg/mL) were added. The incubation was continued for another hour. After washing, cells were treated with 100 μL of diluted rabbit complement at 37°C for 4 h. The negative control groups, 11 μL of DPBS was used to replace the nanobodies. The cell maximum killing was achieved by adding 100 μL of 1% Triton X-100 to the cell culture. The cell viability was measured using a CCK8 assay kit on a microplate reader at 450 nm wavelength. As another control, the cells were also treated with heat inactive complement (complement was kept in 56°C for 30 min) instead of normal complement, so as to confirm that the cytotoxicity was indeed complement-dependent. Specific cytotoxicity was calculated by the following equation:

$$\%\ \text{CDC} = \left(1 - \frac{\text{A (experimental)} - \text{A (maximum)}}{\text{A (negative)} - \text{A (maximum)}}\right) \times 100$$

Where A(negative) is the OD_{450} value of cells treated with DPBS and anti-DNP IgG antibody;

A(experimental) is the OD_{450} value of cells treated with 7D12-DNP conjugates or nanobody 7D12 and anti-DNP IgG antibody; A(maximum) is the OD_{450} value of cells completely lysed with 1% Triton X-100.

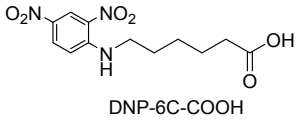
Preparation of DNP-OVA and DNP-HSA.



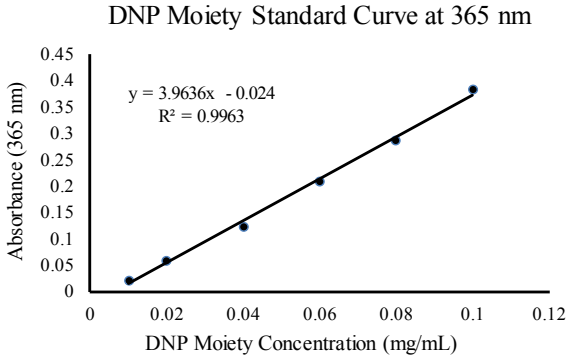
DNP-6C-OSU

2 mg DNP-6C-OSU was dissolved in 100 ul DMSO solution and then it was added to a 2 mL solution of OVA or HSA (20 mg in 0.1 M $NaHCO_3$ solution), respectively. After being shaken at r.t. for 3 days, the conjugates were purified by 10 KDa centrifugal filter devices from the solution. The conjugate fractions were further lyophilized as yellow solids.

The loading of DNP moiety in these conjugates were determined by spectrophotometry. DNP moiety has the maximum absorbance at 365 nm which is used for measuring the DNP loading in conjugates. According to the standard curve made from the DNP-6C-COOH, the DNP loading of DNP-OVA and DNP-HSA were calculated as 8.5% and 12%, respectively. Conjugate DNP-OVA (100 μg DNP-OVA per mouse each time) was dissolved in 1× PBS buffer and then mixed with adjuvant Alum (1:1, v/v) to form an emulsion which was used for mouse immunization according to the protocol provided by manufacturer.



DNP-6C-COOH



DNP-6C-COOH and DNP moiety standard curve used for determination of DNP loading in DNP-OVA and DNP-HSA conjugates.

Immunization of mice to prepare anti-DNP serum. All animal procedures were conducted according to the guidelines and protocols approved by the Institutional Animal Care and Use Committee of Jiangnan

University (JN. No20180115b0850710). DNP-OVA conjugate (2 mg/mL) and Alum Adjuvant (Cat. No. 77161, Thermo, 1 mL) was mixed to prepare an emulsion. Each emulsion (100 μg of DNP-OVA/mouse/injection) was injected subcutaneously (s.c.) into the abdomen of 5 Balb/c mice (female, 6-8 weeks old, Shanghai Slac Laboratory Animal Co., Ltd.) on day 1, 7, 14 and 21 by the similar protocol reported previously.[2] Blood samples were collected one week after final boost immunization and used to prepare anti-DNP sera according to standard protocols. The serum titers of DNP-specific antibodies were determined by ELISA using DNP-HSA as the capture reagent as described previously.[3]

In vivo pharmacokinetics study. 7D12-DNP conjugates or nanobody 7D12 was incubated first with anti-DNP mouse serum or naive serum (4:1 ratio) at 4 °C overnight. Then, each mixture (50 μL) was injected i.p. into the tail vein of a group of 5 Balb/c mice. Blood samples were collected from these mice at different time points and the corresponding sera were analyzed using ELISA by the procedures described below.

High binding 96-well plates were coated with antigen EGFR (5 μg/mL) at 4°C for 12 h and then at 37°C for 1 h. The plates were washed 3 times with PBST and blocked with BSA at room temperature for 2 h. After washing, the plates were incubated with serum in a dilution of 1:100 at 37°C for 2 h. After washing, HRP-conjugated rabbit anti-myc tag IgG antibodies were added and the plates were kept at 37°C for 1 h. The plates were washed and finally analyzed using the TMB kit.

For the standard curves: High binding 96-well plates were coated with antigen EGFR (5 μg/mL) at 4°C for 12 h and then at 37°C for 1 h. The plates were washed 3 times with PBST and blocked with BSA at room temperature for 2 h. After washing, the plates were incubated with the different concentrations (0.2~10 nM) of nanobody 7D12 or nanobody-DNP conjugates at 37°C for 2 h. After washing, HRP-conjugated goat anti-mouce IgG antibodies were added and the plates were kept at 37°C for 1 h. The plates were washed and finally analyzed using the TMB kit.

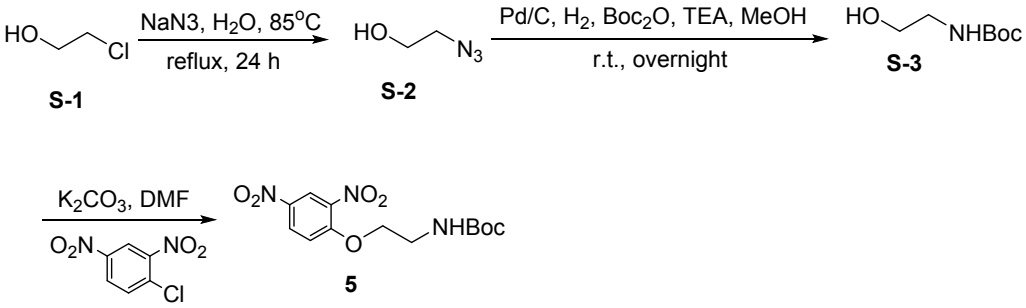
Evaluation of the in vivo antitumor activity using a xenograft model. About 5×10^6 A431 cells suspended in 100 μL of PBS were injected s.c. into the flanks of 15 SCID mice (female, 6-8 weeks-old). Approximately 7 days after solid tumors (100-300 mm^3 in size) were establish, the mice were randomly divided into three groups. Group 1 mice were treated with PBS (50 μL, i.p.) and anti-DNP mouse serum (50 μL, i.p.); Group 2 mice were treated with nanobody 7D12 (40 μg, i.p.) and anti-DNP mouse serum (50 μL, i.p.); Group 3 mice were treated with conjugate **2** (40 μg, i.p.) and anti-DNP mouse serum (50 μL, i.p.). The treatment was

performed 3 times per week for 2 weeks. Tumor size and mouse body weight measurements were taken every 2-3 days. Tumor size was measured with a vernier caliper, and the tumor volume was calculated as: tumor volume = $1/2 \times \text{length} \times \text{width}^2$. After 2 weeks of treatment, the mice were euthanized and their tumors were dissected for weight measurement.

Statistical analysis. The MFI, cell cytotoxicity and serum half-life, and tumor size and weight results were analyzed by Student's t-test or two-way ANOVA. P value of < 0.05 was regarded as statistically significant.

3. Synthesis Procedures for DNP derivatives 13-16

Synthesis of tert-butyl (2-(2,4-dinitrophenoxy)ethyl)carbamate 5:

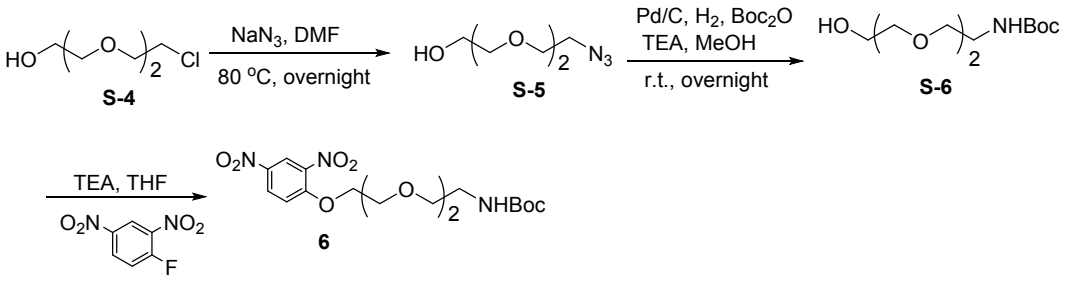


2-azidoethanol S-2: A mixture of 2-Chloroethanol **S-1** (2.0 g, 1 eq), sodium azide (8.0 g, 5 eq) in H_2O was warmed up to 85°C and refluxed for 24 h. After removing the excess sodium azide by filtration, the product was purified by extraction using DCM and evaporated to give 1.9 g **S-2** with 90% yield. The ^{1}H NMR is identical with previous report.[4]

tert-butyl (2-hydroxyethyl)carbamate S-3: Compound **S-2** (1.4 g, 1 eq) and Pd/C (280 mg, 20%) were dissolved in MeOH. Then Boc_2O (5.4 g, 1.5 eq) and TEA (2.7 mL, 1.2 eq) were added. The reaction mixture was stirred overnight at r.t. under hydrogen. The Pd/C was filtered off, washed with DCM, the filtrate was concentrated under reduce pressure. The residue was then purified by column chromatography (DCM/MeOH, 30:1) to provide 2.46 g **S-3** with 95% yield. The ^{1}H NMR is identical with previous report. [5]

tert-butyl (2-(2,4-dinitrophenoxy)ethyl)carbamate 5: To a solution of **S-3** (2.0 g, 1 eq) in DMF, K_2CO_3 (2.37 g, 1.5 eq) and 2,4,-dinitro-1-chlorobenzene (3.46 g, 1.5 eq) were added. The reaction mixture was warmed up to 70°C and stirred for 15 h. The reaction mixture was washed with brine, followed by 3-5 times extraction with DCM. The organic phase was collected and concentrated under reduce pressure. The residue was then purified by column chromatography (DCM/MeOH, 30:1) to give 2.2 g **5** with 60% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.71 (d, *J* = 2.8 Hz, 1H), 8.37 (dd, *J* = 9.2, 2.8 Hz, 1H), 7.20 – 7.05 (m, 1H), 4.24 (t, *J* = 5.0 Hz, 2H), 3.56 (dd, *J* = 11.0, 5.3 Hz, 2H), 1.38 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.53 (s), 155.89 (s), 140.40 (s), 138.85 (s), 129.26 (s), 122.04 (s), 114.60 (s), 80.07 (s), 70.02 (s), 39.59 (s), 28.34 (s). MS: Calculated MW, 327.30; observed, 350.05 $[M+Na]^+$.

Synthesis of tert-butyl (2-(2-(2-(2,4-dinitrophenoxy)ethoxy)ethoxy)ethyl)carbamate 6:

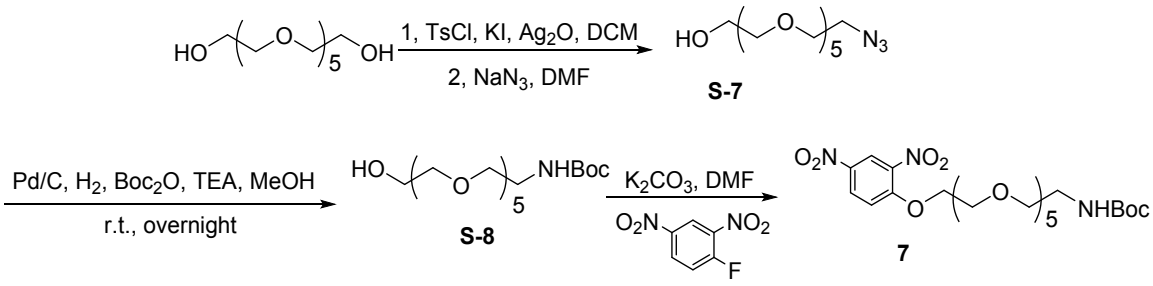


2-(2-(2-azidoethoxy)ethoxy)ethan-1-ol S-5: A mixture of 2-(2-(2-chloroethoxy)ethoxy)ethanol **S-4** (1.2 g, 1 eq), sodium azide (2.3 g, 5 eq) in DMF was stirred overnight at 80°C. After removing the excess sodium azide by filtration, the solution was evaporated and the product was purified via column chromatography (ethyl acetate) to give 1.02 g **S-5** with 80% yield. The ^{1}H NMR was identical with previous report.[6]

***t*-Butyl *N*-2-(2-(2-hydroxyethoxy)ethoxy)ethyl)carbamate S-6:** Compound **S-5** (4 g, 1 eq) and Pd/C (800 mg, 20%) were dissolved in MeOH. Then Boc_2O (7.7g, 1.5 eq) and TEA (3.9 mL, 1.2 eq) were added. The reaction mixture was stirred overnight at r.t. under hydrogen. The Pd/C was filtered off, washed with DCM, the filtrate was concentrated under reduce pressure. The residue was then purified by column chromatography (DCM/MeOH, 30:1) to provide 5.4 g **S-6** with 95% yield. The ^{1}H NMR is identical with previous report.[6]

Synthesis of tert-butyl (2-(2-(2-(2,4-dinitrophenoxy)ethoxy)ethoxy)ethyl)carbamate 6: To a solution of **S-6** (2.0 g, 1 eq) in THF, TEA (1.72 mL, 1.5 eq) was added, followed by 2,4-dinitro-1-fluorobenzene (3.0 g, 2 eq). The reaction mixture was warmed up to 70°C and refluxed until completion of the reaction (checked by TLC). The reaction mixture was then concentrated and purified by column chromatography to give 3.0 g **6** with 90% yield. ^{1}H NMR (400 MHz, $CDCl_3$): ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.14 (s, 1H), 3.78 – 3.73 (m, 2H), 3.68 – 3.60 (m, 6H), 3.56 (t, *J* = 5.2 Hz, 2H), 3.33 (s, 2H), 2.50 (s, 2H), 1.45 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.74 (s), 155.92 (s), 140.20 (s), 139.03 (s), 128.97 (s), 121.75 (s), 114.92 (s), 79.18 (s), 71.08 (s), 70.54 (s), 70.24 (d, *J* = 2.8 Hz), 68.98 (s), 40.33 (s), 28.37 (s). MS: Calculated MW, 415.40; observed, 438.13 $[M+Na]^+$.

Synthesis of tert-butyl (17-(2,4-dinitrophenoxy)-3,6,9,12,15-pentaoxaheptadecyl)carbamate 7:



17-azido-3,6,9,12,15-pentaoxaheptadecan-1-ol S-7: To a solution of 3,6,9,12,15-pentaoxaheptadecane-1,17-diol (2 g, 1 eq) in DCM, Ag_2O (5 g, 1.5 eq), TsCl (1.5 g, 1.1 eq) and KI (247 mg, 0.2 eq) were sequentially added at 0°C. The reaction mixture was allowed to warm up to r.t. and stirred under argon until completion of the reaction (checked by TLC). The mixture was then filtered and washed with ethyl acetate. The filtrate was concentrated under reduce pressure to give crude product, which was directly used in next step without purification.

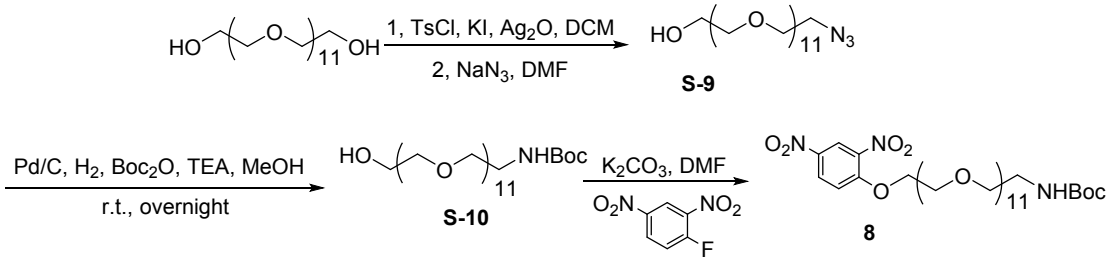
To a solution of the above crude compound in DMF, NaN_3 (2.3 g, 5 eq) was added. The reaction mixture was allowed to warm up to 50°C and stirred overnight. The reaction mixture was then washed with brine. With the subsequent 3-5 times extraction with DCM, the organic phase was collected and concentrated under reduce pressure. The residue was purified by column chromatography (DCM/MeOH, 10:1) to give 1.6 g **S-7** with 77% yield (two steps). The ^{1}H NMR is identical with previous report.[7]

***t*-Butyl (17-hydroxy-3,6,9,12,15-pentaoxaheptadecyl)carbamate S-8:** Compound **S-7** (1.25 g, 1 eq) and Pd/C (125 mg, 10%) were dissolved in MeOH. Then Boc_2O (1.1 g, 1.2 eq) and TEA (0.7 mL, 1.2 eq) were added. The reaction mixture was stirred overnight at r.t. under hydrogen. The Pd/C was filtered off, washed with DCM, the filtrate was concentrated under reduce pressure. The residue was then purified by column chromatography (DCM/MeOH, 10:1) to provide 1.27 g **S-8** with 82% yield. The ^{1}H NMR is identical with previous report.[6]

Synthesis of tert-butyl (17-(2,4-dinitrophenoxy)-3,6,9,12,15-pentaoxaheptadecyl)carbamate 7: To a solution of **S-8** (190 mg, 1 eq) in DMF, K_2CO_3 (50 mg, 1.2 eq) was added, followed by 2,4-dinitro-1-fluorobenzene (61 mg, 1.1 eq). The reaction mixture was warmed up to 80°C and stirred for 16 h. The reaction mixture was washed with brine, followed by 3-5 times extraction with DCM. The organic phase was collected and concentrated under reduce pressure. The residue was then purified by column chromatography (DCM/MeOH, 20:1) to give 160 mg **7** with 67% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.74 (d,

J = 2.8 Hz, 1H), 8.43 (dd, *J* = 9.3, 2.8 Hz, 1H), 7.32 (d, *J* = 9.3 Hz, 1H), 4.45 – 4.38 (m, 2H), 3.98 – 3.92 (m, 2H), 3.75 – 3.70 (m, 2H), 3.64 (dd, *J* = 11.7, 1.9 Hz, 14H), 3.54 (t, *J* = 5.1 Hz, 2H), 3.31 (d, *J* = 5.0 Hz, 2H), 1.44 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.82 (s), 156.01 (s), 140.15 (s), 139.02 (s), 128.97 (s), 121.68 (s), 115.15 (s), 79.06 (s), 71.06 (s), 70.50 (dd, *J* = 6.0, 3.2 Hz), 70.19 (d, *J* = 3.6 Hz), 69.03 (s), 40.35 (s), 28.39 (s). MS: Calculated MW, 547.56; observed, 570.25 $[M+Na]^+$.

Synthesis of tert-butyl (1-(2,4-dinitrophenoxy)-3,6,9,12,15,18,22,25,28,31,34-undecaoxahexatriacontan-36-yl)carbamate 8:



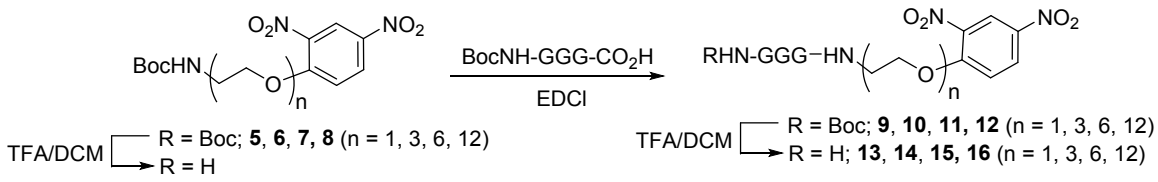
Synthesis of tert-butyl (1-azido-3,6,9,12,15,18,22,25,28,31,34-undecaoxahexatriacontan-36-yl)carbamate S-9: To a solution of 3,6,9,12,15,18,22,25,28,31,34-undecaoxahexatriacontane-1,36-diol (130 mg, 1 eq) in DCM, Ag_2O (82.7 mg, 1.5 eq), TsCl (50.0 mg, 1.1 eq) and KI (8.0 mg, 0.2 eq) were sequentially added at 0°C. The reaction mixture was allowed to warm up to r.t. and stirred under argon until completion of the reaction (checked by TLC). The mixture was then filtered and washed with ethyl acetate. The filtrate was concentrated under reduce pressure to give crude product, which was directly used in next step without purification.

Synthesis of tert-butyl (1-hydroxy-3,6,9,12,15,18,22,25,28,31,34-undecaoxahexatriacontan-36-yl)carbamate S-10: Compound **S-9** (80 mg, 1 eq) and Pd/C (16 mg, 20%) were dissolved in MeOH. Then Boc_2O (41.8 mg, 1.5 eq) and TEA (0.021 mL, 1.2 eq) were added. The reaction mixture was stirred overnight at r.t. under hydrogen. The Pd/C was filtered off, washed with DCM, the filtrate was concentrated under reduce pressure. The residue was then purified by column chromatography (DCM/MeOH, 10:1) to provide 67.8 mg **S-10** with 75% yield. The ^{1}H NMR is identical with previous report.[8]

Synthesis of compound 8: To a solution of **S-10** (190 mg, 1 eq) in DMF, K_2CO_3 (50 mg, 1.2 eq) was added, followed by 2,4-dinitro-1-fluorobenzene (61 mg, 1.1 eq). The reaction mixture was warmed up to 80°C

and stirred for 16 h. The reaction mixture was washed with brine, followed by 3-5 times extraction with DCM. The organic phase was collected and concentrated under reduce pressure. The residue was then purified by column chromatography (DCM/MeOH, 20:1) to give 160 mg **8** with 67% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.74 (d, *J* = 2.8 Hz, 1H), 8.42 (dd, *J* = 9.3, 2.8 Hz, 1H), 7.32 (d, *J* = 9.3 Hz, 1H), 4.45 – 4.37 (m, 2H), 3.97 – 3.91 (m, 2H), 3.76 – 3.70 (m, 2H), 3.67 – 3.60 (m, 38H), 3.54 (t, *J* = 5.1 Hz, 2H), 3.35 – 3.26 (m, 2H), 1.44 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.84 (s), 156.07 (s), 140.23 (s), 139.10 (s), 128.99 (s), 121.79 (s), 115.11 (s), 79.16 (s), 71.14 (s), 70.58 (d, *J* = 6.8 Hz), 70.27 (d, *J* = 5.1 Hz), 69.09 (s), 28.45 (s). HRMS: Calculated MW, 811.8760; observed, 812.4038 $[M+H]^+$.

Synthesis of compounds 13, 14, 15 and 16:



Compound **5**, **6**, **7** or **8** was dissolved in 50% TFA/DCM and the reaction mixture was stirred at r.t. for 2 h. The DCM was removed by reduced pressure. The crude residue was re-dissolved in toluene and concentrated to remove excess TFA under reduce pressure to give product which was used directly without purification.

To the above obtained amine in DCM/DMF (1:2), Boc protected triglycine (1.2 equiv), EDCI (1.2 eq) and DIPEA (1.2 eq) were sequentially added. The reaction mixture was stirred at r.t. until completion of the reaction (checked by TLC). The reaction mixture was then concentrated and sequentially purified by column chromatography to give product **9**-**12**.

9: in 62% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.73 (d, *J* = 2.7 Hz, 1H), 8.43 (dd, *J* = 9.3, 2.8 Hz, 1H), 7.27 (d, *J* = 3.0 Hz, 1H), 4.35 (s, 2H), 3.94 (dd, *J* = 15.7, 5.7 Hz, 4H), 3.83 – 3.68 (m, 4H), 1.41 (s, 9H)..^{13}C NMR (101 MHz, $CDCl_3$) δ 171.13 (s), 169.89 (d, *J* = 17.6 Hz), 156.71 (s), 156.50 (s), 140.36 (s), 138.68 (s), 129.49 (s), 121.99 (s), 115.02 (s), 80.69 (s), 69.10 (s), 44.43 (s), 43.09 (d, *J* = 7.4 Hz), 38.36 (s), 28.27 (s). HRMS: Calculated MW, 498.4490, observed, 499.1788 $[M+H]^+$.

10: in 60% yield.^{1}H NMR (400 MHz, MeOD): δ 8.72 (d, *J* = 2.8 Hz, 1H), 8.49 (dd, *J* = 9.3, 2.8 Hz, 1H), 7.55 (d, *J* = 9.3 Hz, 1H), 4.50 – 4.45 (m, 2H), 3.95 – 3.90 (m, 4H), 3.87 (s, 2H), 3.77 – 3.71 (m, 4H), 3.64 (dd, *J* = 5.6, 3.5 Hz, 2H), 3.56 (t, *J* = 5.6 Hz, 2H), 3.38 (dd, *J* = 9.3, 3.6 Hz, 2H), 1.46 (s, 9H). ^{13}C NMR (101 MHz, MeOD): δ 172.13 (s),

170.79 (s), 170.20 (s), 157.35 (s), 156.45 (s), 140.18 (s), 139.13 (s), 128.68 (s), 120.95 (s), 115.26 (s), 79.63 (s), 70.59 (s), 70.38 (s), 69.98 (s), 69.01 (s), 68.79 (s), 43.53 (s), 42.36 (s), 42.01 (s), 39.04 (s), 27.31 (s); HRMS: Calculated MW, 586.5550, observed, 587.2308 $[M+H]^+$.

11: in 50% yield.^{1}H NMR (400 MHz, MeOD): δ 8.72 (d, *J* = 2.8 Hz, 1H), 8.48 (dd, *J* = 9.3, 2.8 Hz, 1H), 7.55 (d, *J* = 9.4 Hz, 1H), 4.49 – 4.43 (m, 2H), 3.90 (dd, *J* = 12.7, 10.2 Hz, 6H), 3.77 – 3.50 (m, 20H), 3.37 (dd, *J* = 11.4, 5.9 Hz, 2H), 1.45 (s, 9H). ^{13}C NMR (101 MHz, MeOD): δ 172.21 (s), 170.85 (s), 170.26 (s), 162.09 (s), 156.47 (s), 140.16 (s), 139.18 (s), 128.65 (s), 120.92 (s), 115.32 (s), 79.62 (s), 70.49 (d, *J* = 19.5 Hz), 70.07 (dd, *J*= 16.3, 11.3 Hz), 69.01 (s), 68.79 (s), 43.52 (s), 42.36 (s), 41.99 (s), 39.06 (s), 27.30 (s); HRMS: Calculated MW, 718.7140, observed, 719.3086 $[M+H]^+$.

12: in 16% yield. ^{1}H NMR (400 MHz, MeOD) δ 8.72 (d, *J* = 2.8 Hz, 1H), 8.48 (dd, *J* = 9.3, 2.8 Hz, 1H), 7.56 (d, *J* = 9.4 Hz, 1H), 4.49 – 4.43 (m, 2H), 3.99 – 3.84 (m, 6H), 3.77 – 3.59 (m, 42H), 3.55 (s, 2H), 3.38 (t, *J* = 5.5 Hz, 2H), 1.45 (s, 9H). ^{13}C NMR (101 MHz, MeOD) δ 172.02 (s), 170.75 (s), 170.19 (s), 156.48 (s), 128.66 (s), 120.91 (s), 115.40 (s), 79.56 (s), 70.52 (d, *J* = 16.5 Hz), 70.17 (d, *J* = 7.7 Hz), 69.92 (s), 68.82 (s), 43.52 (s), 42.34 (s), 42.02 (s), 39.06 (s), 27.34 (s). MS: Calculated MW, 983.03, observed, 984.33 $[M+H]^+$.

Compound **9, 10, 11** or **12** was dissolved in 50% TFA/DCM and the reaction mixture was stirred at r.t. for 2 h. The DCM was removed by reduced pressure. The crude residue was re-dissolved in toluene and concentrated to remove excess TFA under reduce pressure to give product **13**-**16**.

13: 95% yield. ^{1}H NMR (400 MHz, D_2O) δ 8.87 (d, *J* = 2.7 Hz, 1H), 8.53 (dd, *J* = 9.4, 2.7 Hz, 1H), 7.47 (d, *J* = 9.4 Hz, 1H), 4.46 (t, *J* = 5.0 Hz, 2H), 4.08 (s, 2H), 3.95 (d, *J* = 7.2 Hz, 4H), 3.73 (t, *J* = 5.0 Hz, 2H). ^{13}C NMR (101 MHz, D_2O) δ 171.61 (d, *J* = 14.7 Hz), 167.86 (s), 156.77 (s), 139.93 (s), 138.08 (s), 130.10 (s), 122.26 (s), 115.58 (s), 68.90 (s), 42.43 (d, *J* = 11.1 Hz), 40.49 (s), 38.22 (s). HRMS: Calculated MW, 398.1186; observed, 399.1279 $[M+H]^+$.

14: 95% yield. ^{1}H NMR (400 MHz, MeOD): δ 8.72 (d, *J* = 2.8 Hz, 1H), 8.48 (dd, *J* = 9.3, 2.8 Hz, 1H), 7.54 (d, *J* = 9.4 Hz, 1H), 4.72 – 4.35 (m, 2H), 3.97 (s, 2H), 3.92 (dd, *J* = 5.0, 3.7 Hz, 2H), 3.86 (s, 2H), 3.79 – 3.70 (m, 4H), 3.65 – 3.60 (m, 2H), 3.55 (t, *J* = 5.6 Hz, 2H), 3.36 (dd, *J* = 10.2, 4.6 Hz, 2H). ^{13}C NMR (101 MHz, MeOD): δ 170.30 (s), 170.08 (s), 166.78 (s), 156.43 (s), 140.19 (s), 139.11 (s), 128.69 (s), 120.97 (s), 115.21 (s), 70.56 (s), 70.34 (s), 69.88 (s), 69.05 (s), 68.75 (s), 42.00 (d, *J* = 13.7 Hz), 40.13 (s), 38.98 (s). HRMS: Calculated MW, 486.4380, observed, 487.1792 $[M+H]^+$.

15: 95% yield. ^{1}H NMR (400 MHz, MeOD): δ 8.74 (d, *J* = 2.7 Hz, 1H), 8.49 (dd, *J* = 9.3, 2.8 Hz, 1H), 7.55 (d, *J* = 9.3 Hz, 1H), 4.50 – 4.44 (m, 2H), 3.96 – 3.89 (m, 4H), 3.87 (s, 2H), 3.80 (s, 2H), 3.74 (dd, *J* = 5.4, 3.1 Hz, 2H), 3.68 – 3.57 (m, 16H), 3.39 – 3.33 (m, 2H). ^{13}C NMR (101 MHz, MeOD): δ 170.44 (s), 161.87 (s), 156.40 (s), 140.25 (s), 139.13 (s), 128.74 (s), 120.98 (s), 115.32 (s), 70.48 (s), 70.43 – 69.72 (m), 69.39 (s), 68.72 (s), 42.67 (s), 42.06 (s), 40.22 (s), 38.78 (s); HRMS: Calculated MW, 618.5970, observed, 619.2580 $[M+H]^{+}$.

16: 95% yield. ^{1}H NMR (400 MHz, MeOD) δ 8.74 (d, *J* = 2.8 Hz, 1H), 8.51 (dd, *J* = 9.3, 2.8 Hz, 1H), 7.57 (d, *J* = 9.3 Hz, 1H), 4.53 – 4.44 (m, 2H), 3.94 (dd, *J* = 5.2, 3.6 Hz, 4H), 3.88 (d, *J* = 4.1 Hz, 4H), 3.72 – 3.61 (m, 42H), 3.41 (t, *J* = 5.1 Hz, 2H). ^{13}C NMR (101 MHz, MeOD) δ 170.43 (s), 167.54 (s), 156.45 (s), 140.19 (s), 139.16 (s), 128.68 (s), 120.93 (s), 115.35 (s), 70.48 (d, *J* = 15.3 Hz), 70.22 – 69.42 (m), 69.29 (s), 68.74 (d, *J* = 5.4 Hz), 42.84 (s), 42.22 (s), 40.48 (s), 38.83 (s), 31.66 (s), 29.34 (s). MS: Calculated MW, 882.92, observed, 883.41 $[M+H]^{+}$.

4. References

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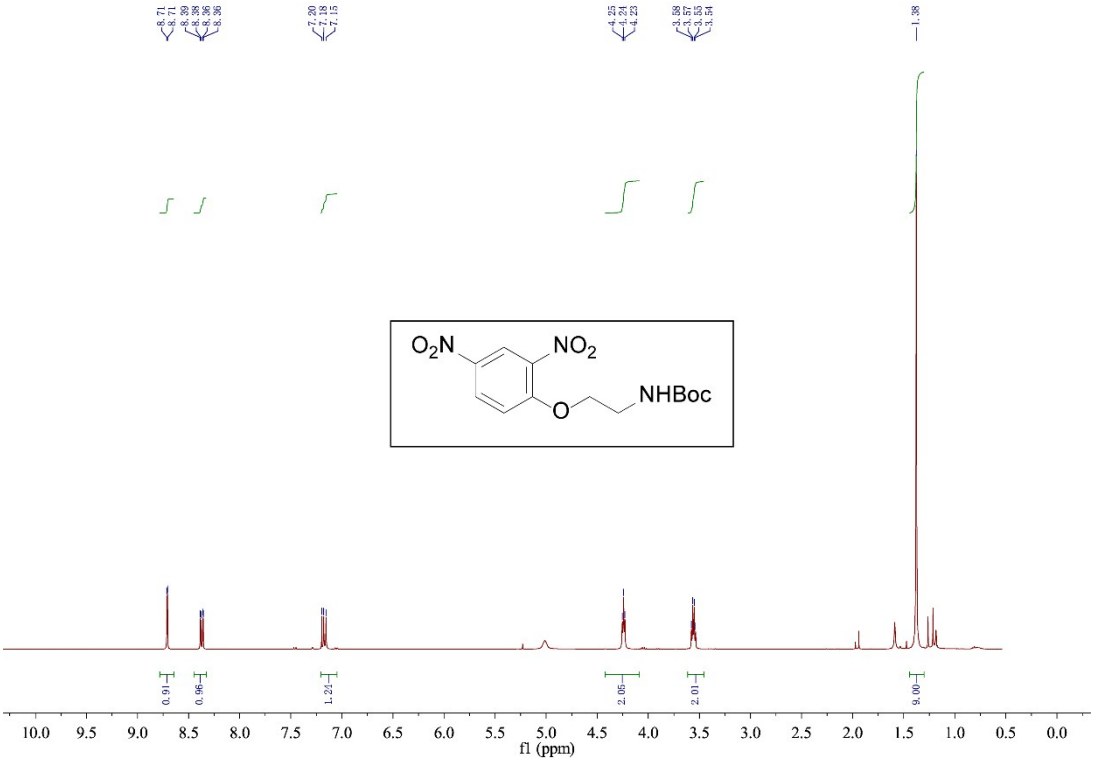
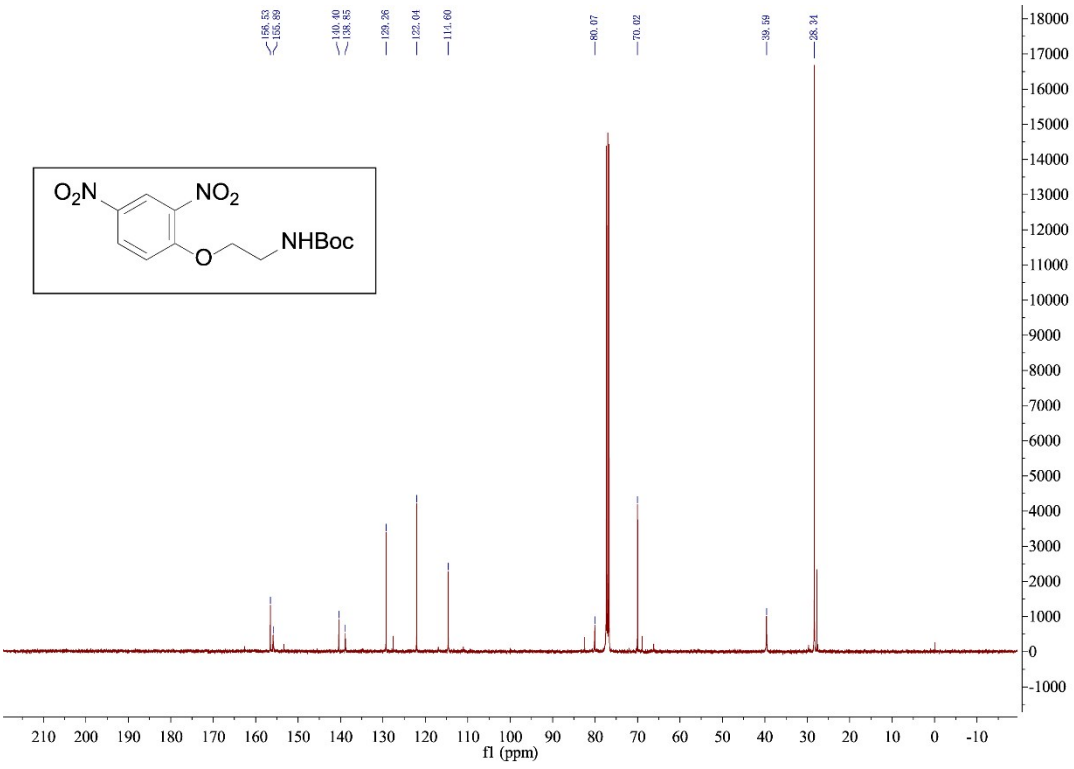
5. 1H-and 13C-NMR Spectra

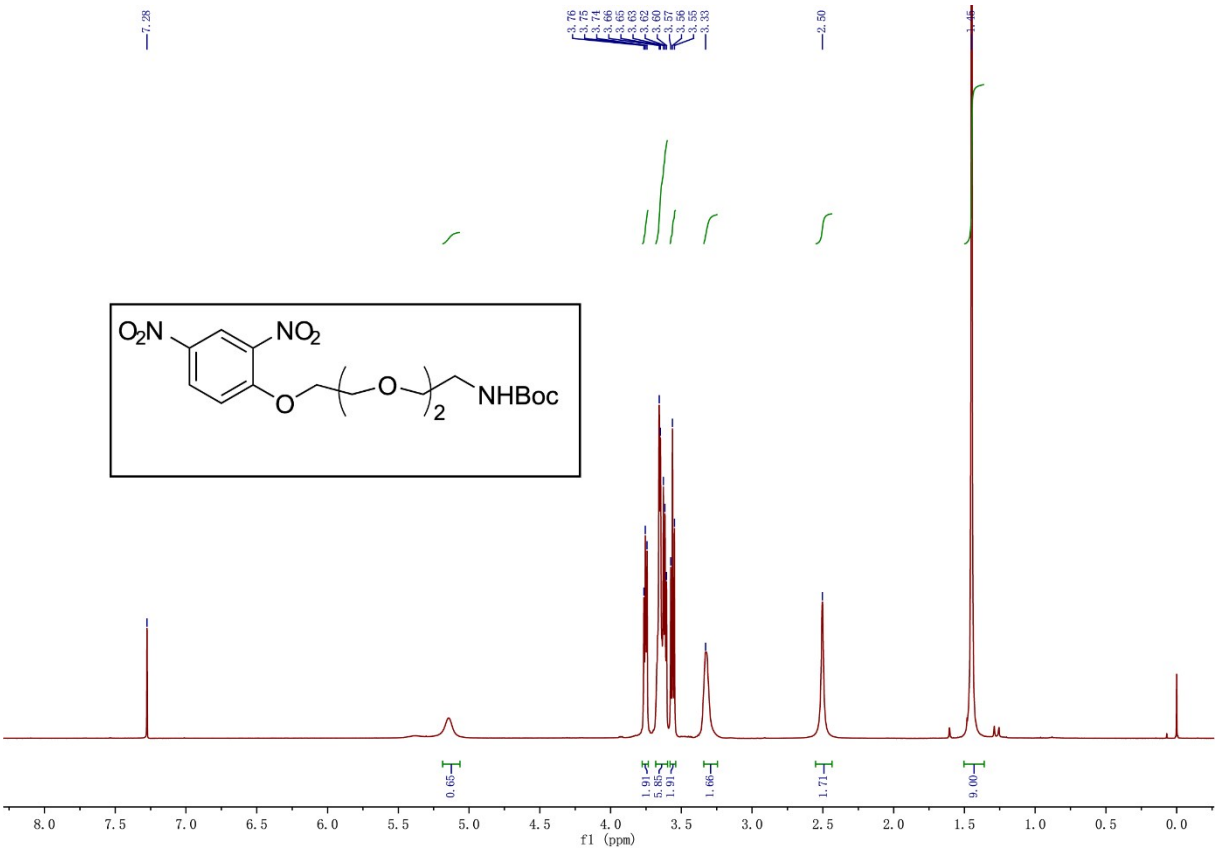
^{1}H-NMR spectrum of compound **5** in $CDCl_3$.



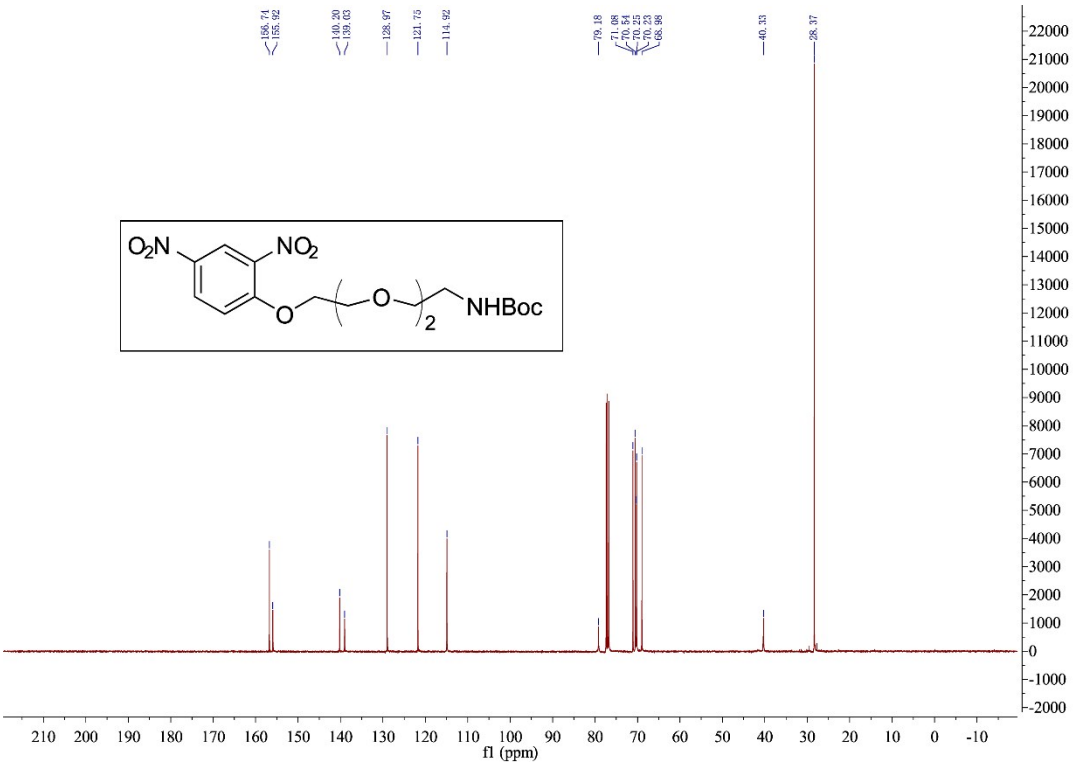
^{13}C-NMR spectrum of compound **5** in $CDCl_3$.



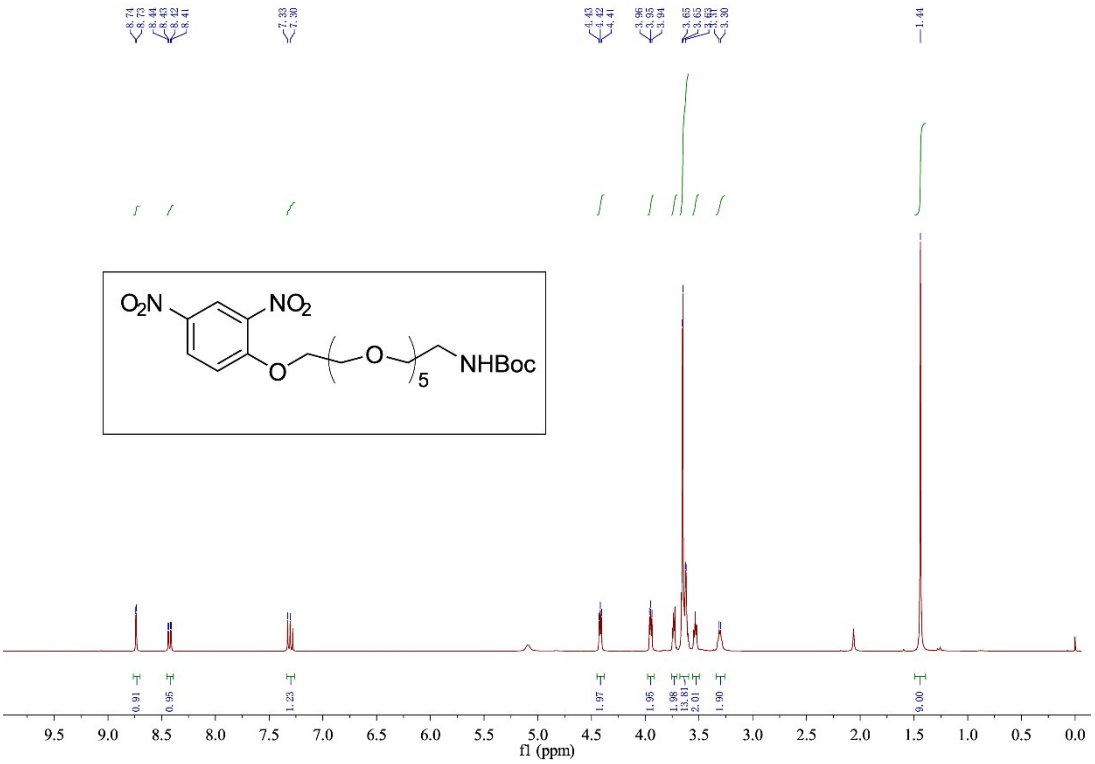
^{1}H-NMR spectrum of compound **6** in $CDCl_3$.



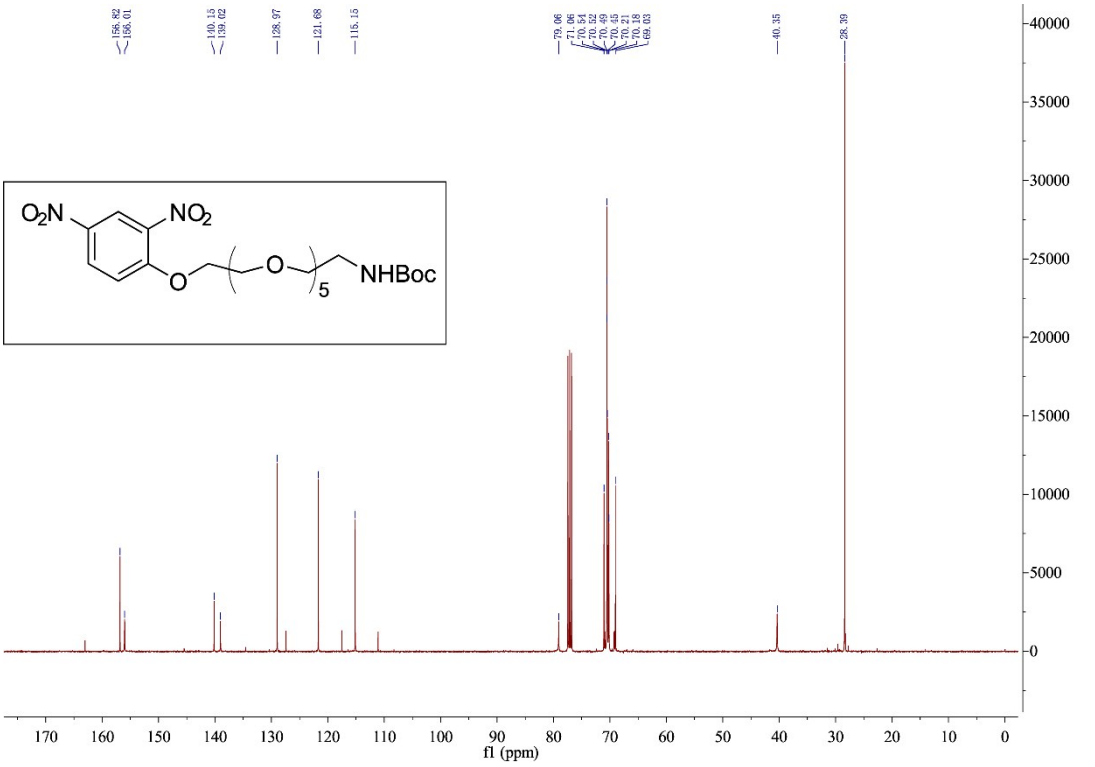
^{13}C-NMR spectrum of compound **6** in $CDCl_3$.



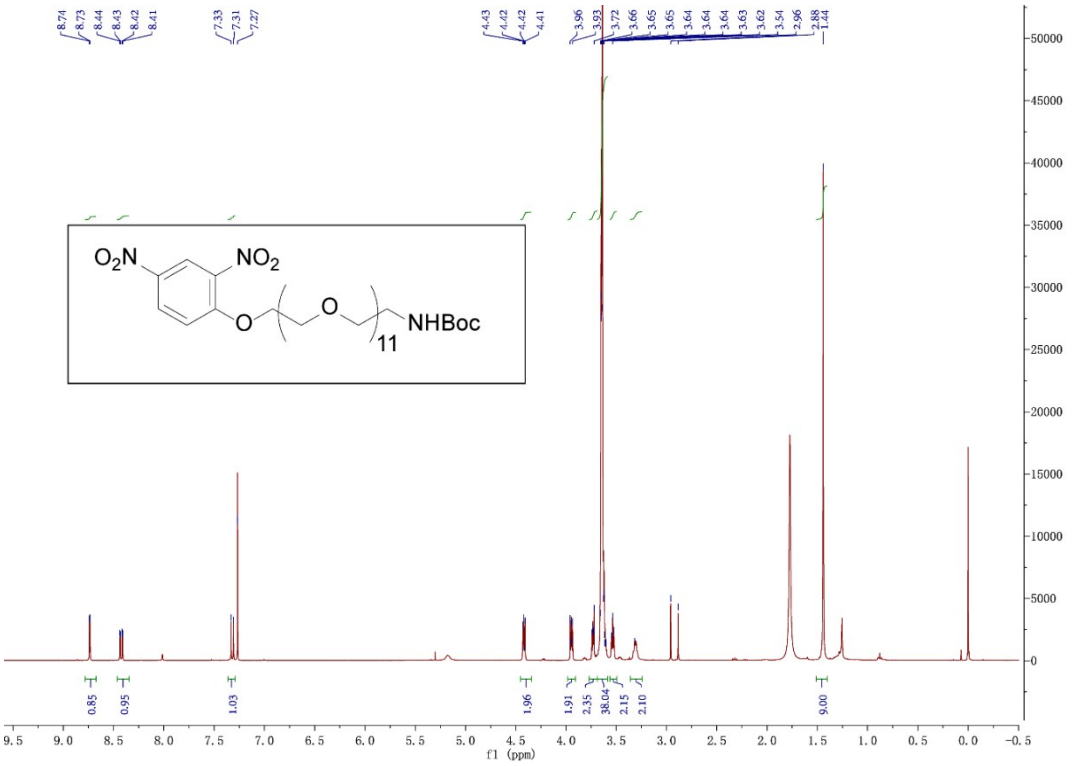
^{1}H-NMR spectrum of compound **7** in $CDCl_3$.



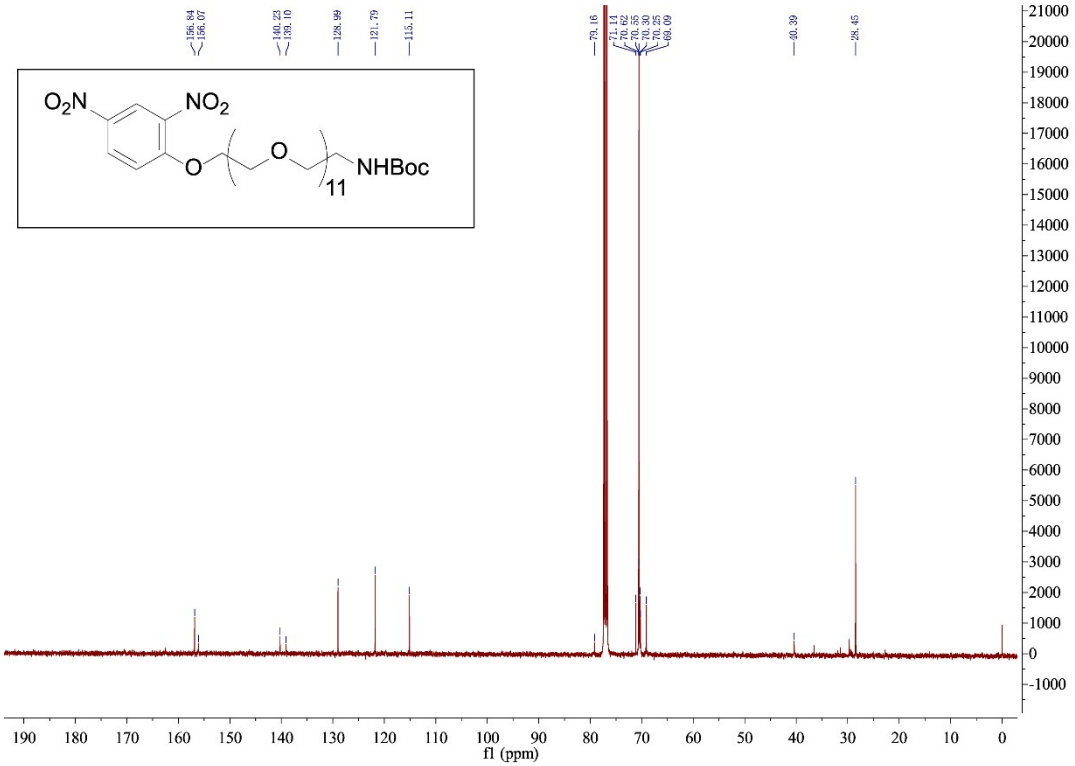
^{13}C-NMR spectrum of compound **7** in $CDCl_3$.



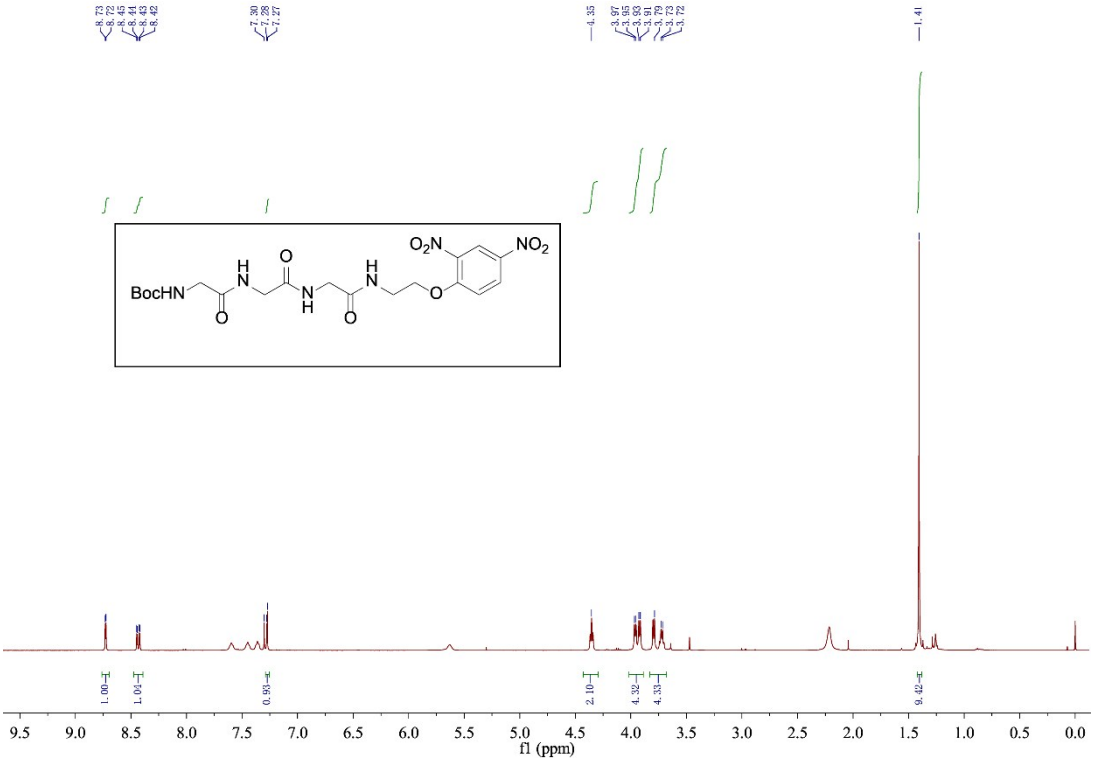
^{1}H-NMR spectrum of compound **8** in $CDCl_3$.



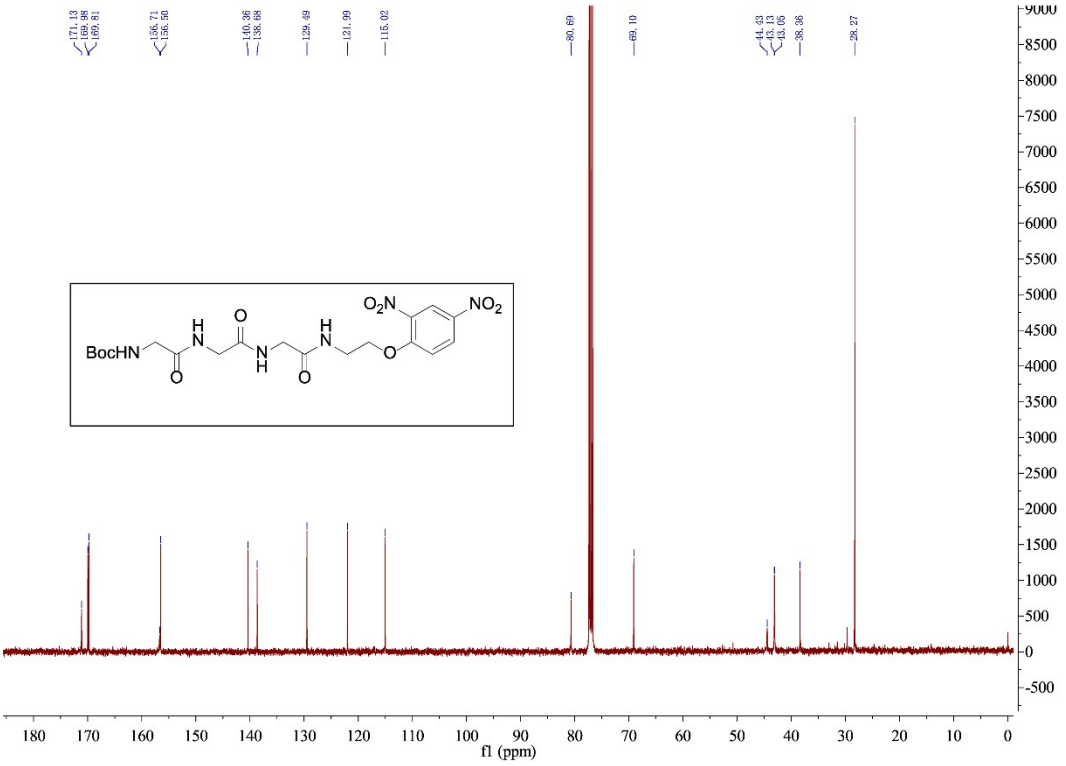
^{13}C-NMR spectrum of compound **8** in $CDCl_3$.



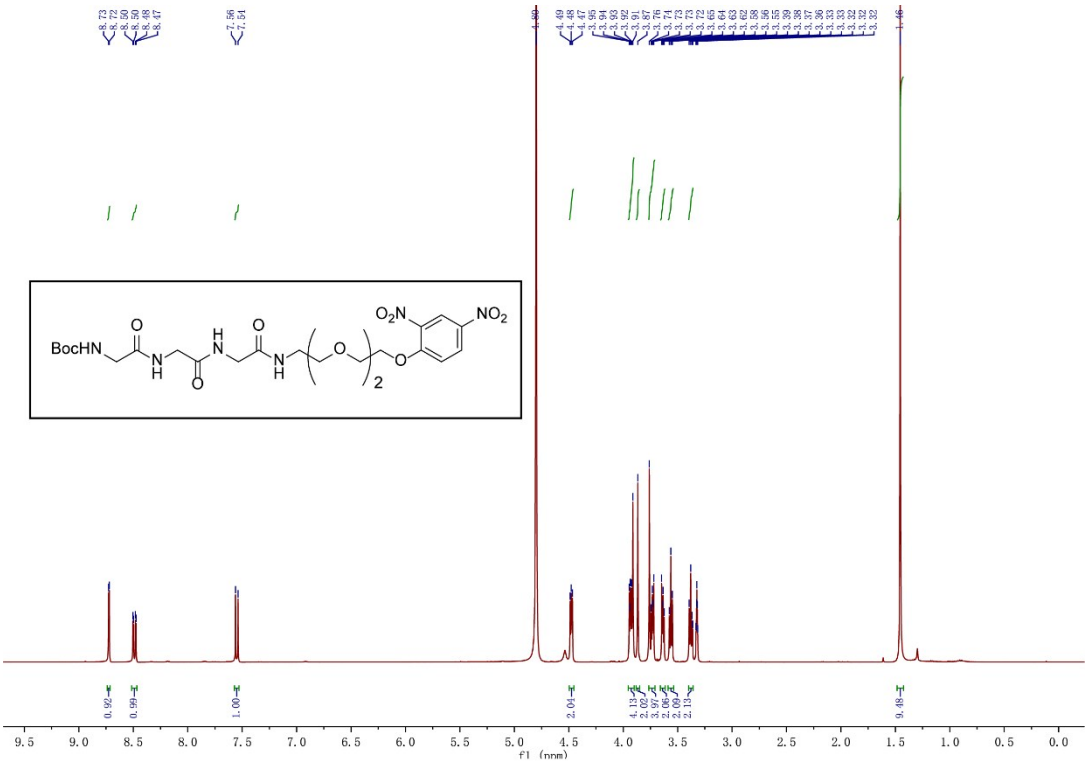
^{1}H-NMR spectrum of compound **9** in $CDCl_3$.



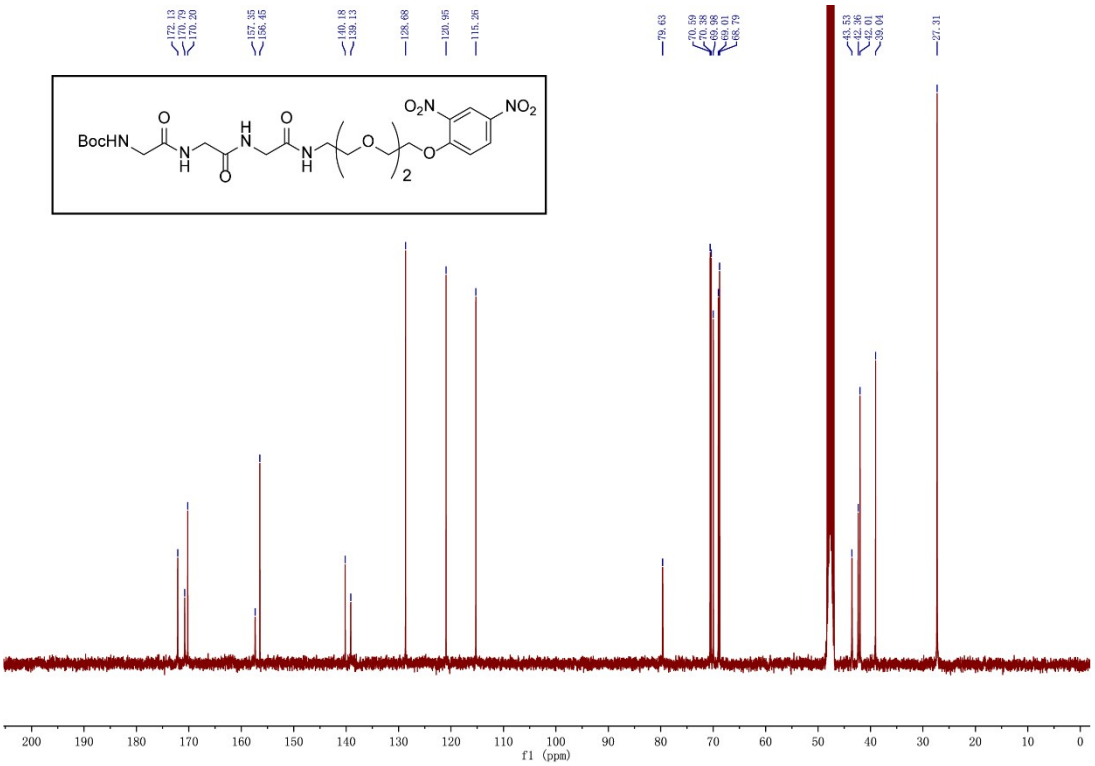
^{13}C-NMR spectrum of compound **9** in $CDCl_3$.



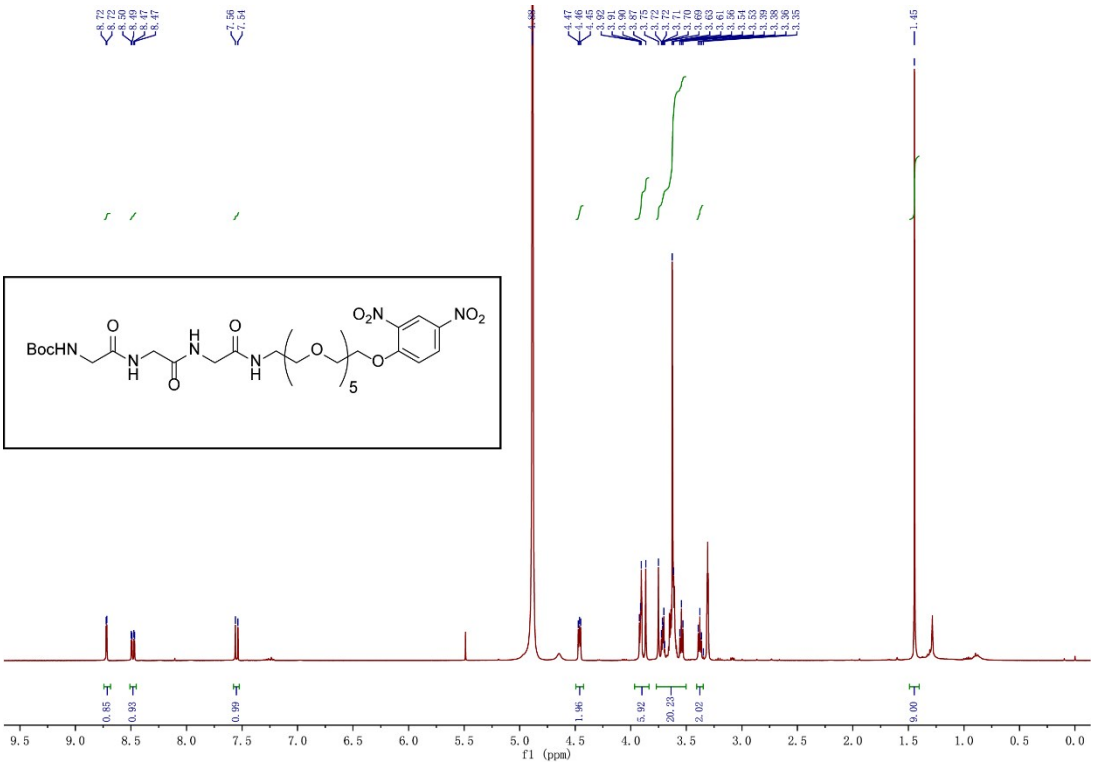
^{1}H-NMR spectrum of compound **10** in MeOD.



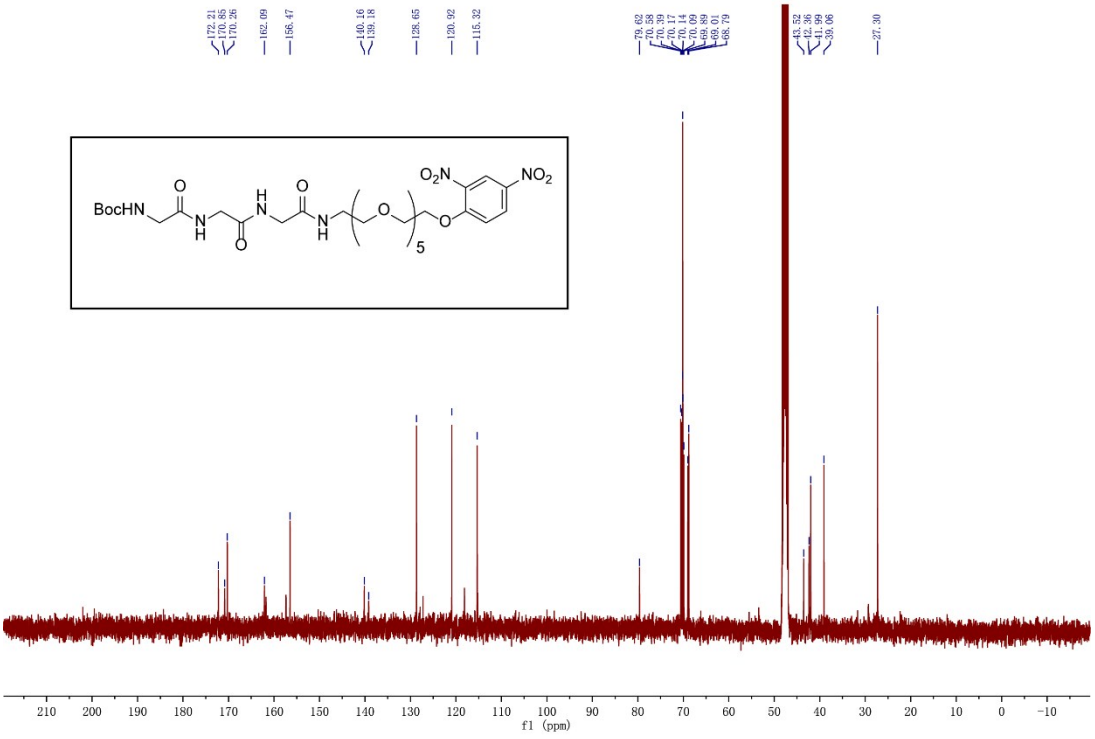
^{13}C-NMR spectrum of compound **10** in MeOD.



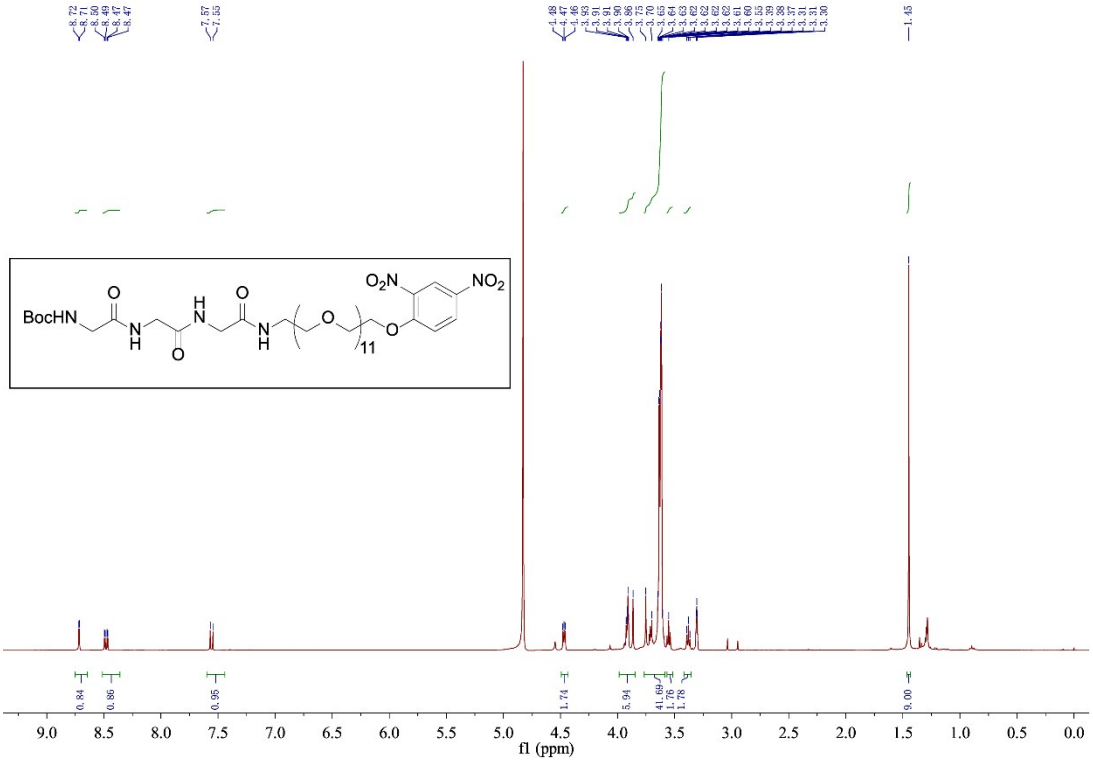
^{1}H-NMR spectrum of compound **11** in MeOD.



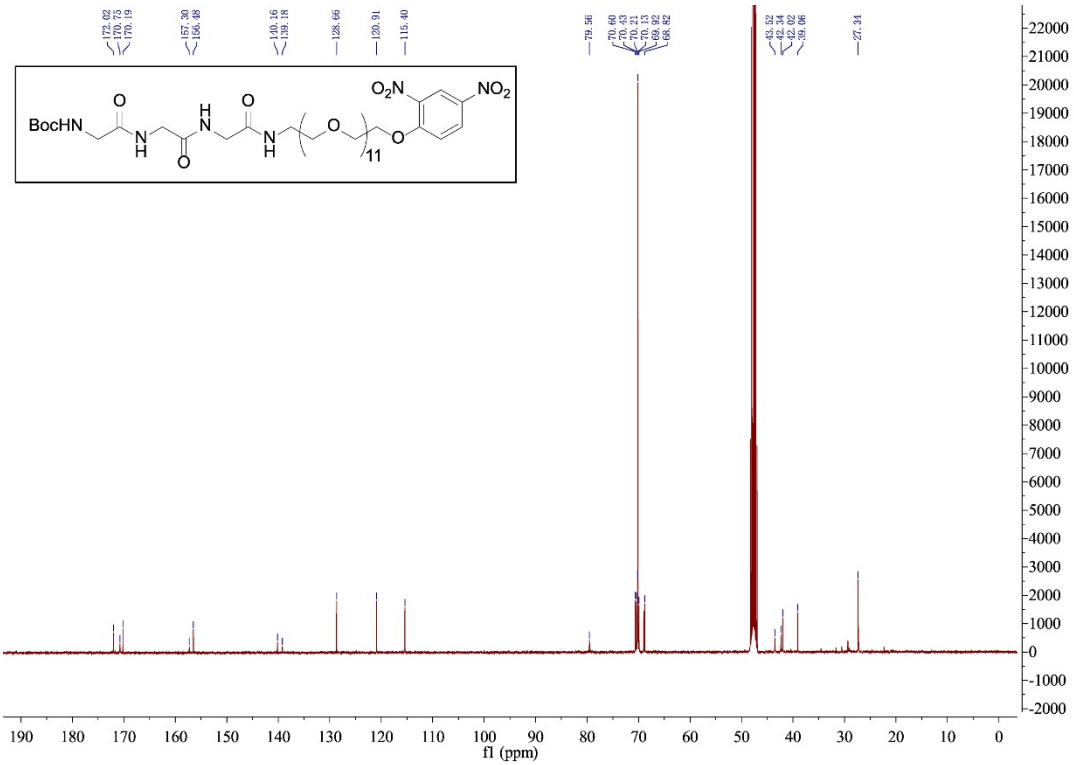
^{13}C-NMR spectrum of compound **11** in MeOD.



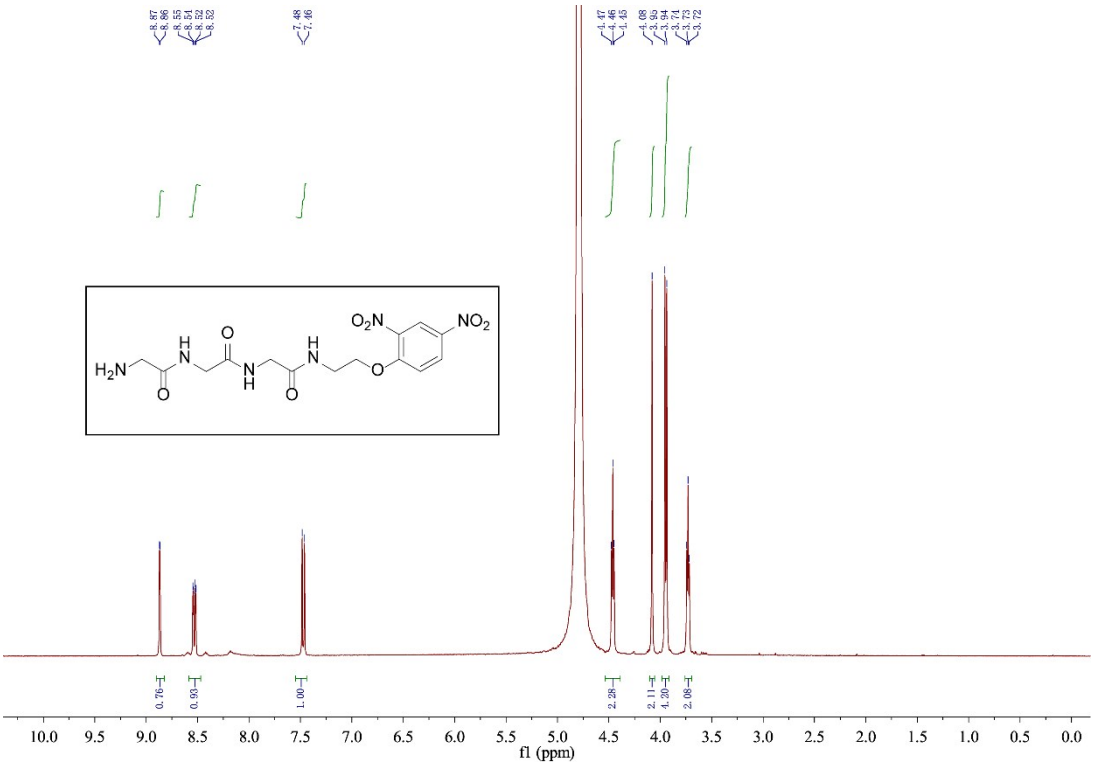
^{1}H-NMR spectrum of compound **12** in MeOD.



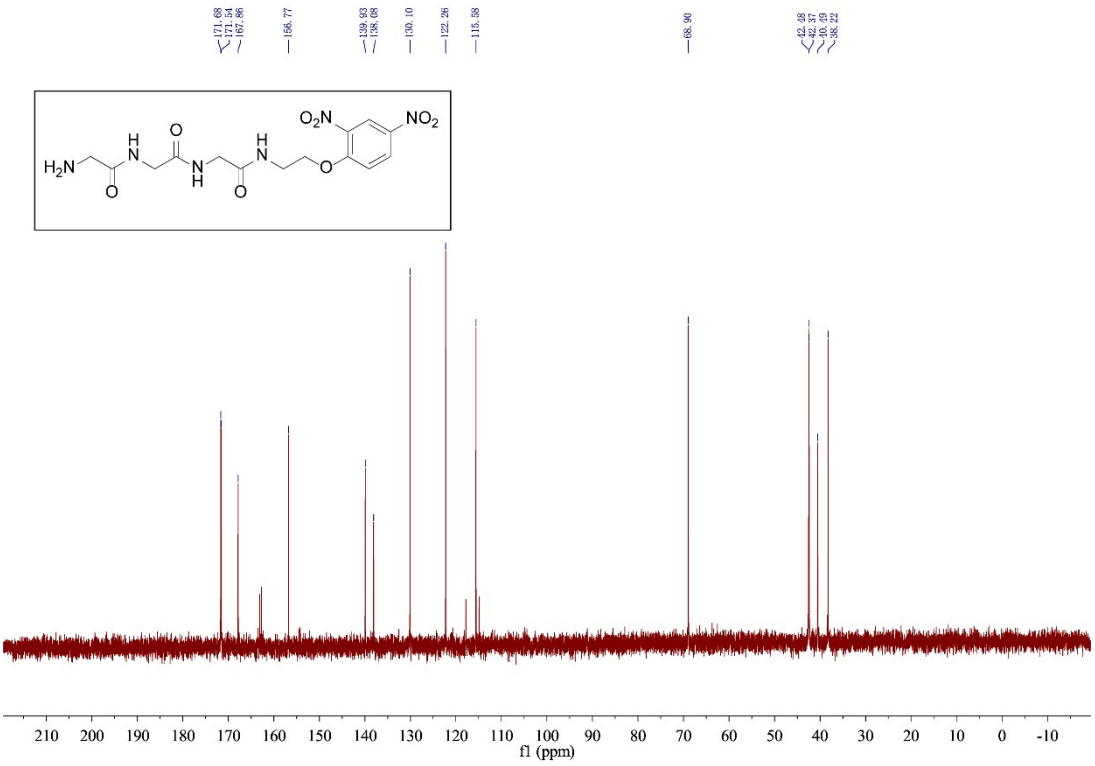
^{13}C-NMR spectrum of compound **12** in MeOD.



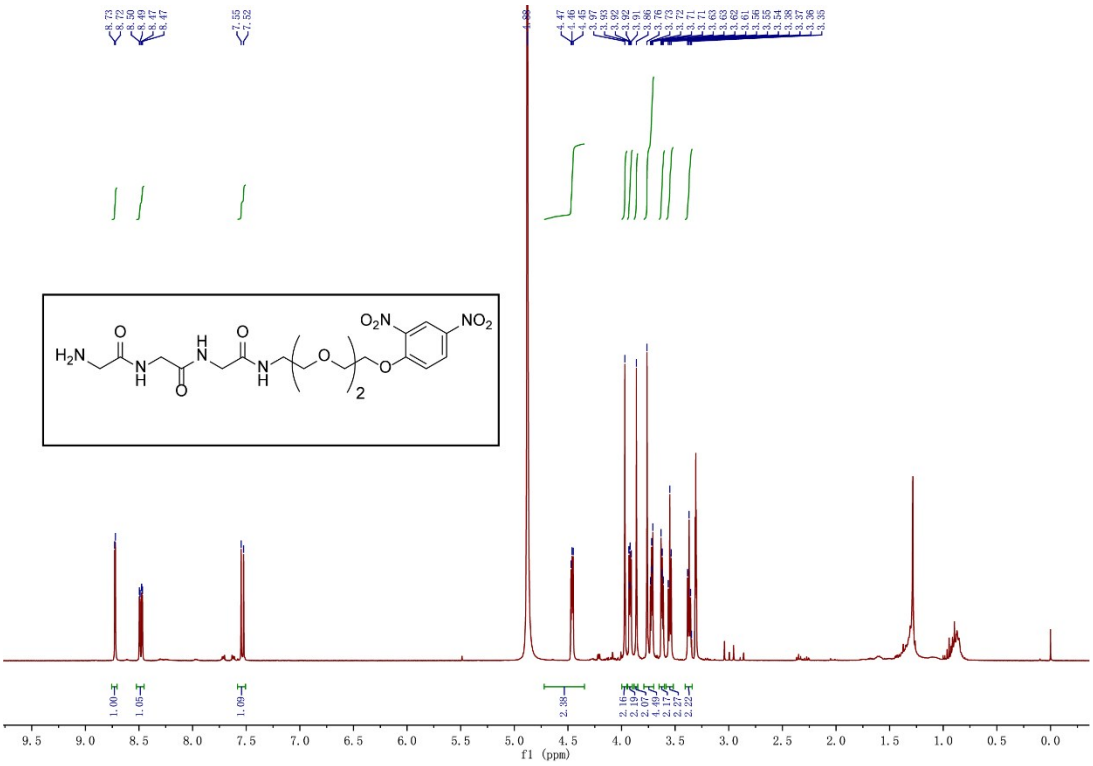
^{1}H-NMR spectrum of compound **13** in D_2O.



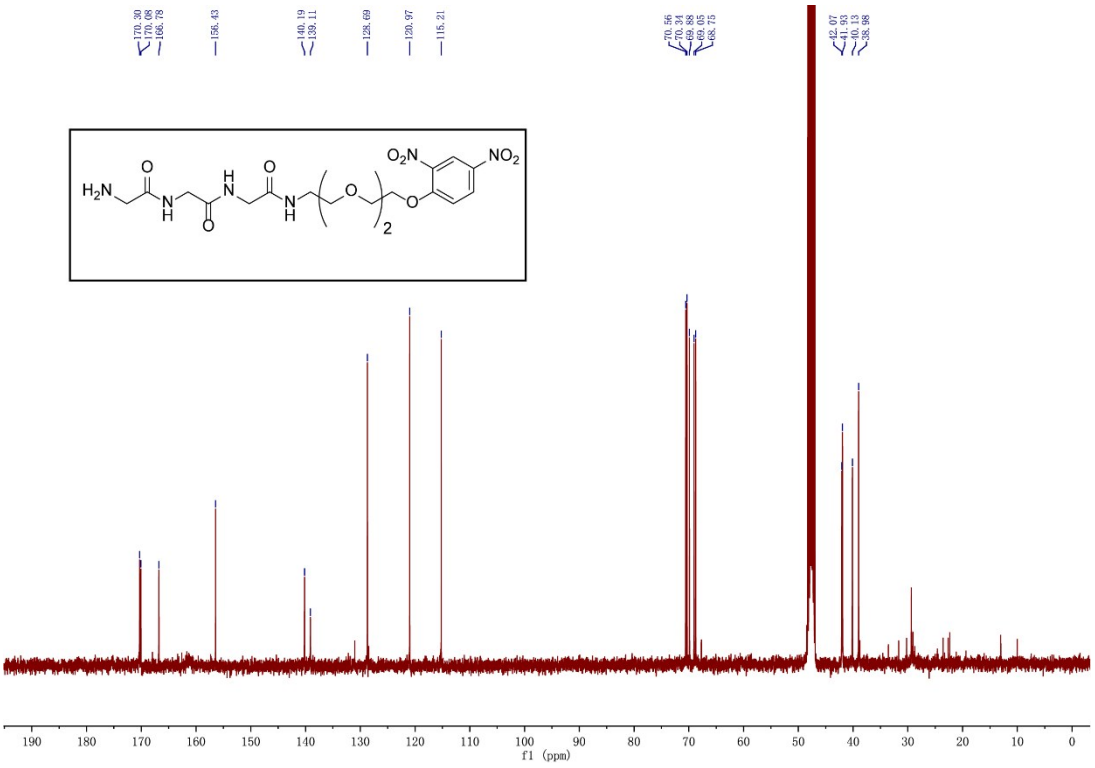
^{13}C-NMR spectrum of compound **13** in D_2O.



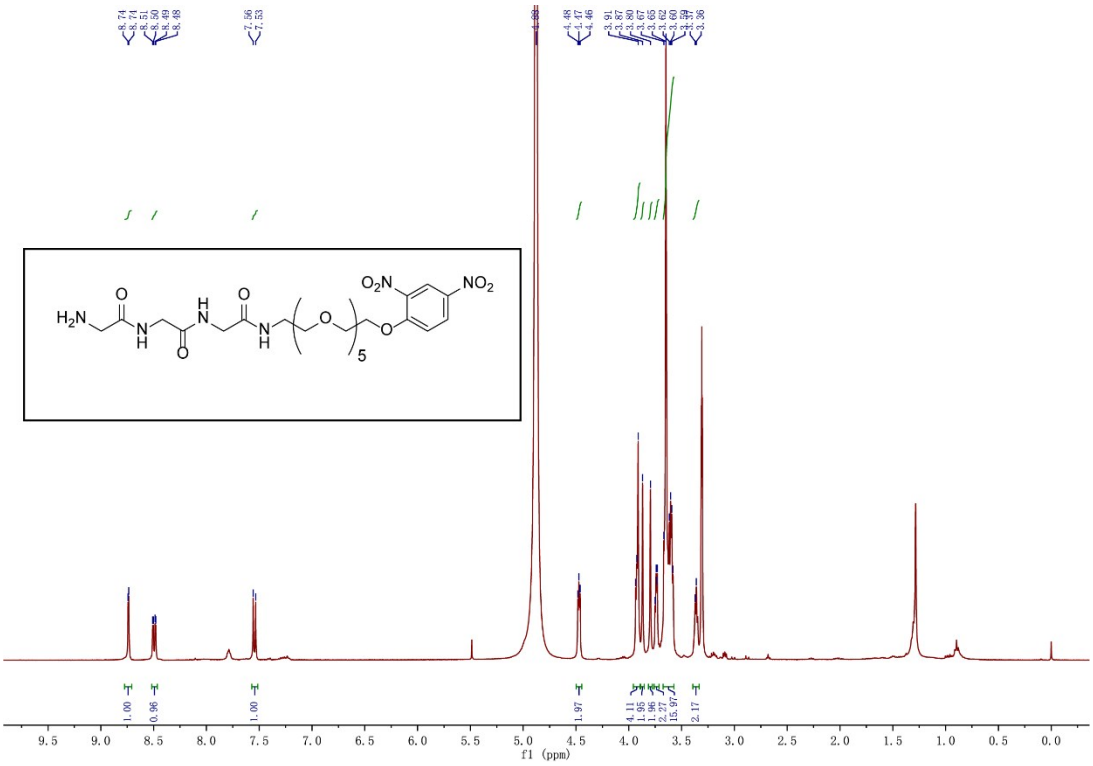
^{1}H-NMR spectrum of compound **14** in MeOD.



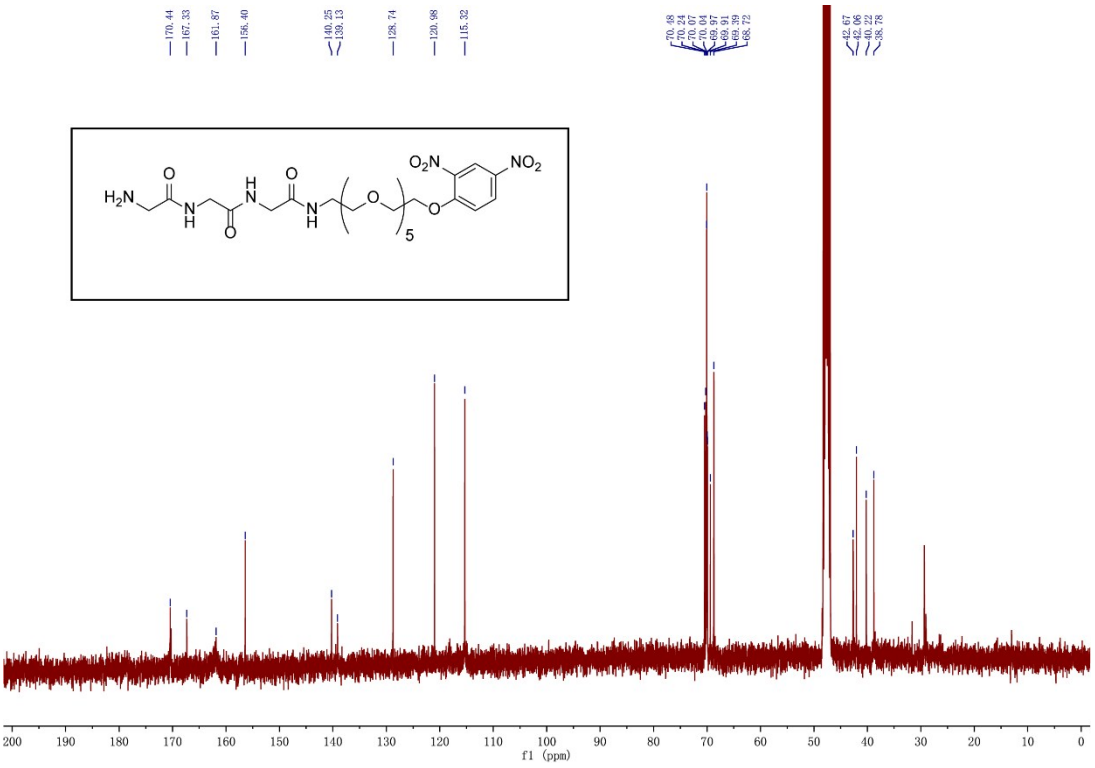
^{13}C-NMR spectrum of compound **14** in MeOD.



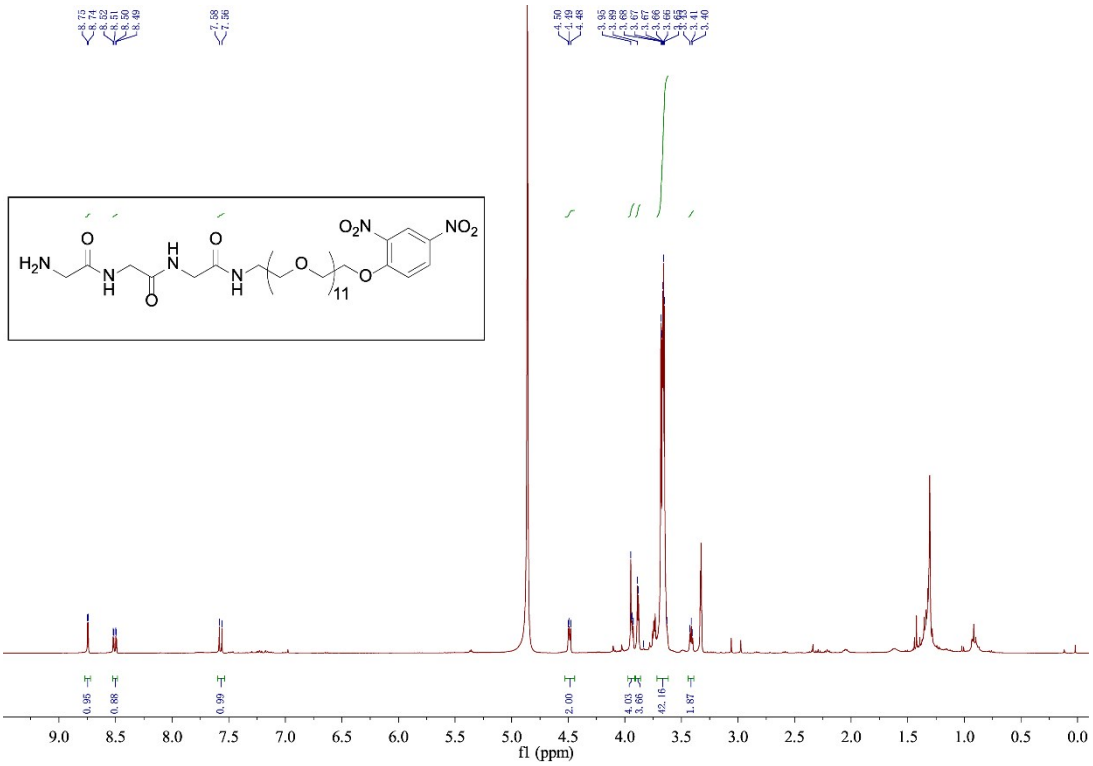
^{1}H-NMR spectrum of compound **15** in MeOD.



^{13}C-NMR spectrum of compound **15** in MeOD.



^{1}H-NMR spectrum of compound **16** in MeOD.



^{13}C-NMR spectrum of compound **16** in MeOD.