Supplementary information

An antibody–supermolecule conjugate for tumor-specific targeting of tumoricidal methylated β-cyclodextrin-threaded polyrotaxanes

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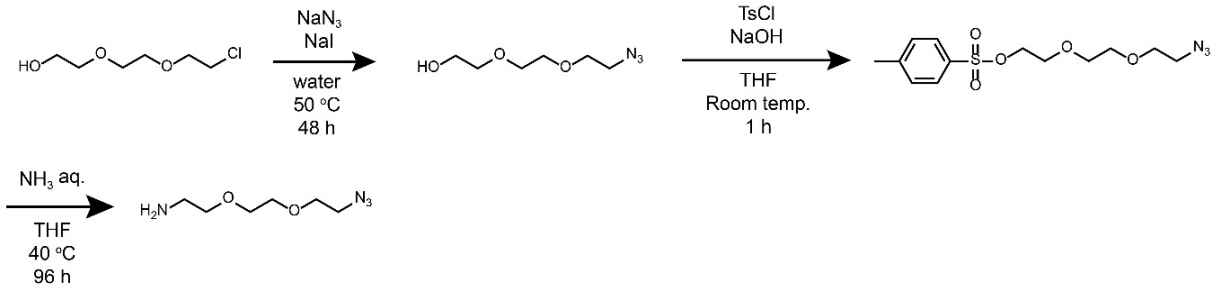
S1. Materials

Sodium azide (NaN_3), sodium iodide (NaI), *p*-toluenesulfonyl chloride (TsCl), ammonia solution (28%), maleic anhydride, 4-ethynylaniline, and acetic anhydride were purchased from Fujifilm Wako Pure Chemical (Osaka, Japan). 2-[2-(2-Hydroxyethoxy)ethoxy]ethyl chloride and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Anhydrous sodium acetate was purchased from Kanto Chemicals (Tokyo, Japan). Dibenzocyclooctyne (DBCO)-fluorescein isothiocyanate (FITC) conjugate (DBCO-FITC; product name: DBCO Fluor 488) was purchased from Click Chemistry Tools (Macon, GA, USA).

S2. Synthesis of 2-[2-(2-azidoethoxy)ethoxy]ethanamine



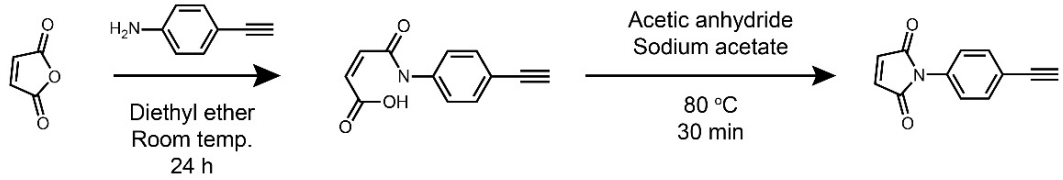
Scheme S1. Scheme for the synthesis of 2-(2-(2-azidoethoxy)ethoxy)ethanamine.

2-[2-(2-Azidoethoxy)ethoxy]ethanamine was synthesized according to previous report with slight modifications (**Scheme S1**). 2-[2-(2-Hydroxyethoxy)ethoxy]ethyl chloride (15 g, 88.9 mmol), NaI (4.06 g, 27.1 mmol), and NaN_3 (45.3 mg, 697 mmol) were dissolved in distilled water (90 mL), and the solution was stirred for 48 h at 50 °C. The reaction mixture extracted with ethyl acetate for three times. The organic layer was dried over Na_2SO_4, and evaporated to collect 2-[2-(2-azidoethoxy)ethoxy]ethanol (14.0 g, 90.1%) as a yellow oil.

To the solution 2-[2-(2-azidoethoxy)ethoxy]ethanol (8 g, 45.7 mmol) in THF (22.4 mL), 6 M NaOH (20.8 mL) and TsCl (13.1 g, 68.6 mmol) were added at 0 °C. The reaction mixture was stirred for 1 h at room temperature under nitrogen atmosphere. The solution was then extracted with diethyl ether four times, and the organic layer was washed with 1 M NaOH. After evaporation of the organic layer, 2-[2-(2-azidoethoxy)ethoxy]ethyl tosylate (16.8 g, 98.6%) was obtained as a yellow oil.

Finally, ammonia solution (28%, 14.8 mL, 365 mol) was added to the 2-[2-(2-azidoethoxy)ethoxy]-ethyl tosylate (8 g, 24.3 mmol) dissolved in THF (95.9 mL), and reaction mixture was stirred for 96 h at 40 °C. After the reaction, distilled water (50 mL) was added to the solution. The mixture was washed with diethyl ether and then extracted with dichloromethane. The organic layer was dried over $NaSO_4$ and evaporated to obtain 2-[2-(2-azidoethoxy)ethoxy]ethanamine (2.49 g, 58.8%) as a yellow oil.

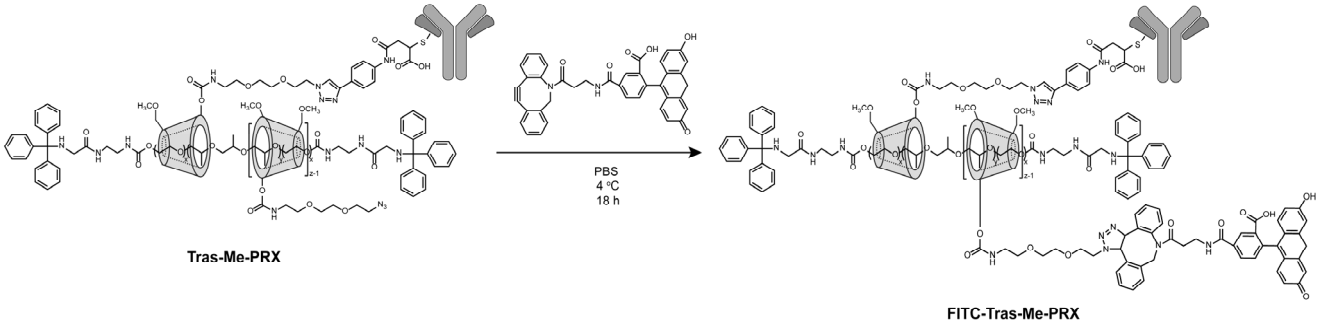
S3. Synthesis of *N*-(4-ethynylphenyl)maleimide



Scheme S2. Scheme for the synthesis of *N*-(4-ethynylphenyl)maleimide.

Maleic anhydride (1.84 g, 19.0 mmol) and 4-ethynylaniline (2.0 g, 16.8 mmol) were dissolved in diethyl ether (350 mL), and the reaction mixture was stirred for 24 h at room temperature. After the reaction, the obtained dark yellow powder was collected by filtration. The powder was then dissolved in a mixture of acetic anhydride and anhydrous sodium acetate, and the reaction mixture was stirred for 30 min at 80 °C. After cooling to room temperature, ice water was added to the solution. The precipitate was collected by filtration and washed with water. The precipitate was dissolved in chloroform, the solution was dried over $MgSO_4$, and evaporated to recover *N*-(4-ethynylphenyl)maleimide (2.33 g, 70.4%).

S4. Synthesis of the fluorescently labeled trastuzumab-Me-PRX conjugate



Scheme S3. Scheme for the preparation of the fluorescently labeled trastuzumab-Me-PRX conjugate.

DBCO-FITC (2.57 μg) was added to the PBS solution of Tras-Me-PRX (300 μg antibody), and the solution was gently shaken at 4 °C for 18 h. The FITC-labeled Tras-Me-PRX was purified by gel filtration (Sephadex G75) and eluted with PBS (pH 7.4). The number of FITC dye modified on Tras-Me-PRX was determined from the absorbance at 488 nm using a V-550 UV/VIS spectrophotometer (Jasco, Tokyo, Japan).

S5. Quantification of thiol groups in the antibody by 5,5′-dithiobis 2-nitrobenzoic acid (DTNB).
Trastuzumab (4 mg antibody) was dissolved in 1 mL of PBS (pH 7.4, 150 mM NaCl, 20 mM EDTA). The solution was incubated with TCEP at 37 °C for 1.5 h, and subsequently, placed on ice bath for 10 min. The reacted solution was purified using Sephadex G25 to obtain reduced trastuzumab. The solutions (100 μg/mL of antibody) of trastuzumab, reduced trastuzumab, and Tras-Me-PRX were incubated with DTNB (final concentration of 1 mM) at room temperature for 30 min. After the reaction, the absorbance of each solution was measured at 405 nm using a V-550 UV/VIS spectrophotometer. The free thiol group concentration in the antibody was determined from the calibration curve of *N*-acetyl-L-cysteine (1 to 500 μM in PBS), and the number of thiol groups in the antibodies was calculated using the following equation:

$$\text{Number of thiol groups in antibody} = \frac{\text{Concentration of thiol groups in antibody (μM)}}{\text{Concentration of antibody (μM)}}$$

The number of reacted thiol groups in the Tras-Me-PRX was determined by subtracting number of thiol groups in the reduced trastuzumab with that in the Tras-Me-PRX (**Table S1**).

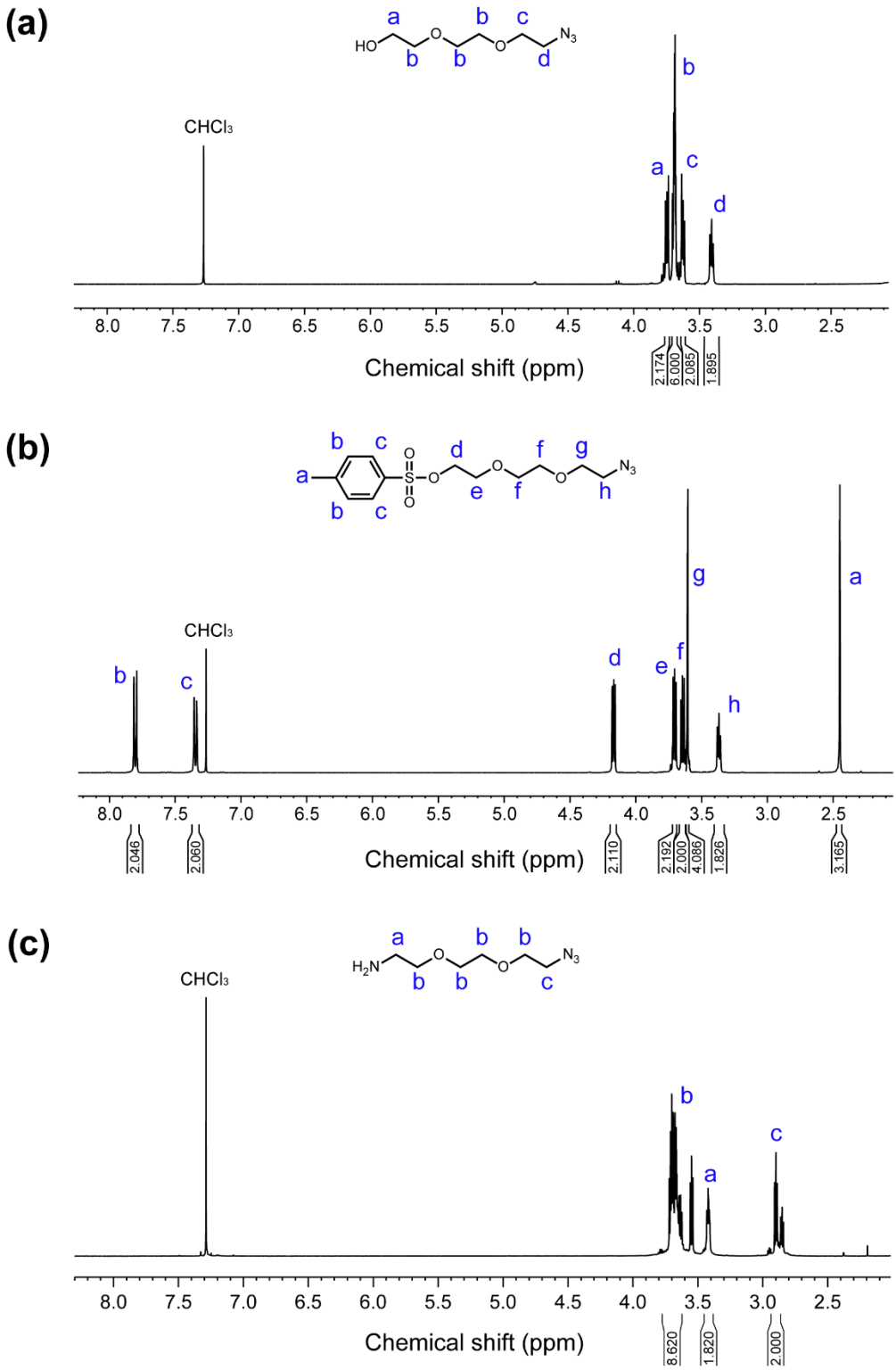


Figure S1. ^{1}H NMR spectra of 2-[2-(2-azidoethoxy)ethoxy]ethanol (a), 2-[2-(2-azidoethoxy)ethoxy]ethyl tosylate (b), and 2-[2-(2-azidoethoxy)ethoxy]ethanamine (c) in $CDCl_3$.

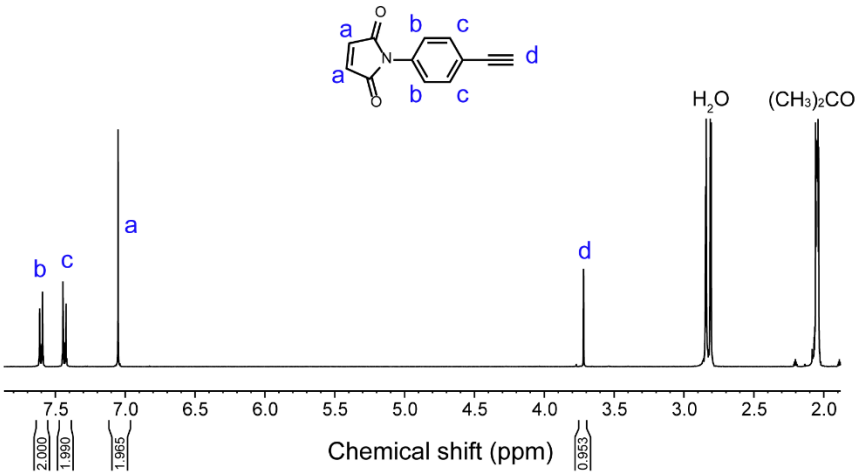


Figure S2. ^{1}H NMR spectrum of *N*-(4-ethynylphenyl)maleimide in acetone-d_6.

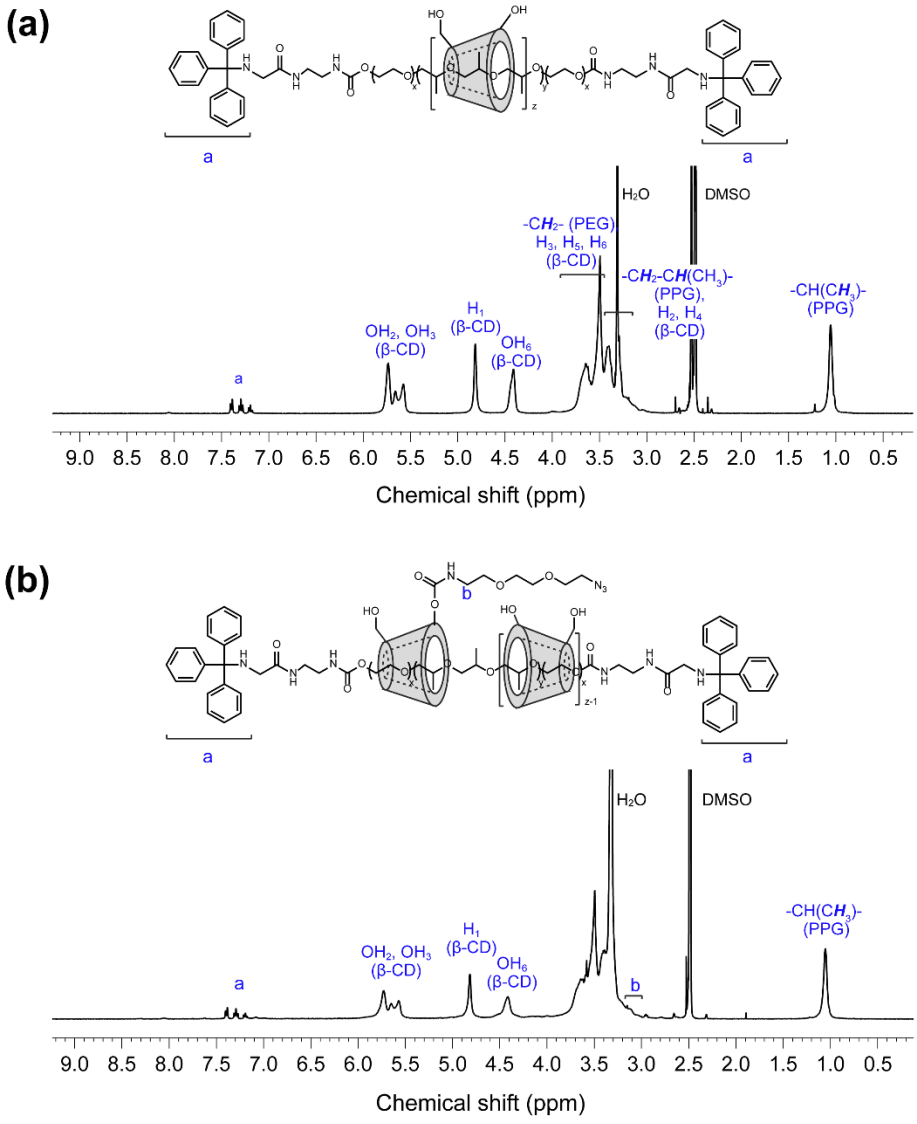


Figure S3. ^{1}H NMR spectra of unmodified PRX (a) and N_3-PRX (b) in DMSO-d_6.

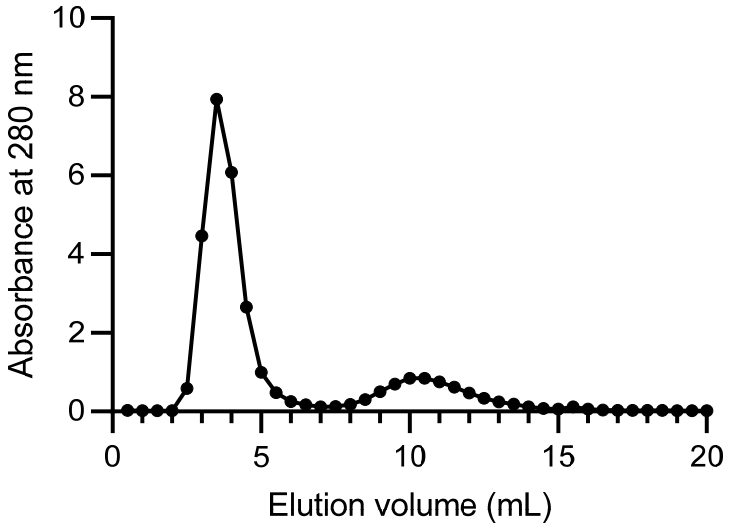


Figure S4. Gel filtration chromatogram of Tras-Me-PRX on Sephadex G75 eluted with PBS containing 20 mM EDTA. Absorbance of each collected fraction was measured at 280 nm.

Table S1. Number of thiol groups in antibodies determined by DTNB.

Samples	Number of thiol groups	Number of reacted thiol groups
Trastuzumab	0.00	-
Reduced trastuzumab	4.65	-
Tras-Me-PRX	0.24	4.41

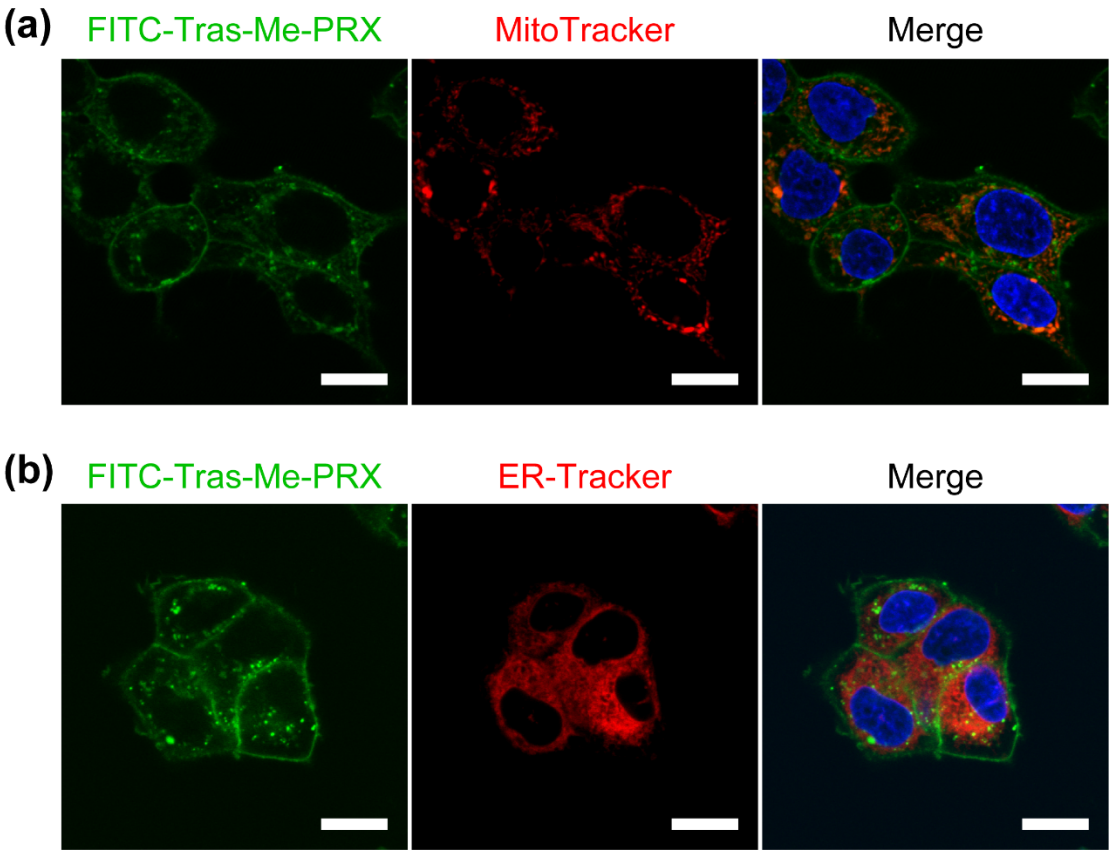


Figure S5. CLSM images of BT-474 and HeLa cells treated with FITC-Tras-Me-PRX (10 μg/mL antibody, 0.27 μM Me-PRX) (green) for 24 h (Scale bars: 20 μm). (a) The nuclei and mitochondria were stained with Hoechst 33342 (blue) and MiroTracker Red (red), respectively. (b) The nuclei and and ER were stained with Hoechst 33342 (blue) and ER-Tracker Red (red), respectively.