Supplementary Information

Dissecting the structural basis for the intracellular delivery of OSW-1 by fluorescent probes

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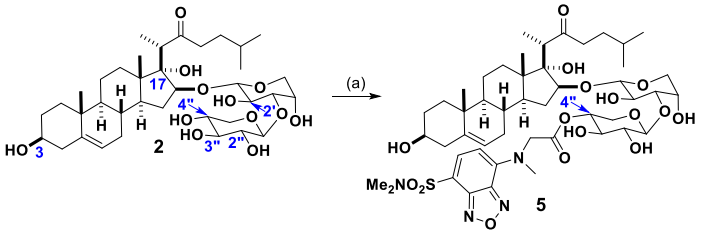
1. General Experimental Methods

NMR experiments were performed at 293 K on JEOL ECX 300, 400 or ECA 500 using 5 mm z-gradient probes and data were processed with Delta software. The spectra are referenced internally according to residual solvent signals of $CDCl_3$ (^{1}H NMR; δ = 7.26 ppm, ^{13}C NMR; δ = 77.0 ppm) and pyridine-d_5 (^{1}H NMR; δ = 8.74 ppm, ^{13}C NMR; δ = 149.9 ppm). Positive ion ESI-TOF-MS data were obtained by JEOL AccuTOF mass spectrometer. Unless noted otherwise, all chemical reagents were purchased from Wako Chemicals, TCI and Sigma-Aldrich. Flash column chromatography was performed using Silica gel 60 (spherical, particle size 40-100 μm; Kanto Chemical). Liquid chromatography was performed using Biotage® SNAP cartridge KP-C18-HS 12 g column (particle size 37-70; Biotage Japan) on Biotage Isolera One system (Biotage Japan, Tokyo). Analytical scale HPLC experiments were performed using a 4.6 × 25.0 mm (5 μm) Waters Xbridge ODS column on JASCO LC-2000 Plus system (Tokyo) equipped with a UV detection unit UV-2075 Plus. UV-visible spectra were recorded on Varian Cary 50 UV-visible spectrophotometer. Fluorescence spectra were recorded on JASCO ETC 273 spectrophotometer.

2. Synthesis of 4”-DBD-deacetylated OSW-1 (5)

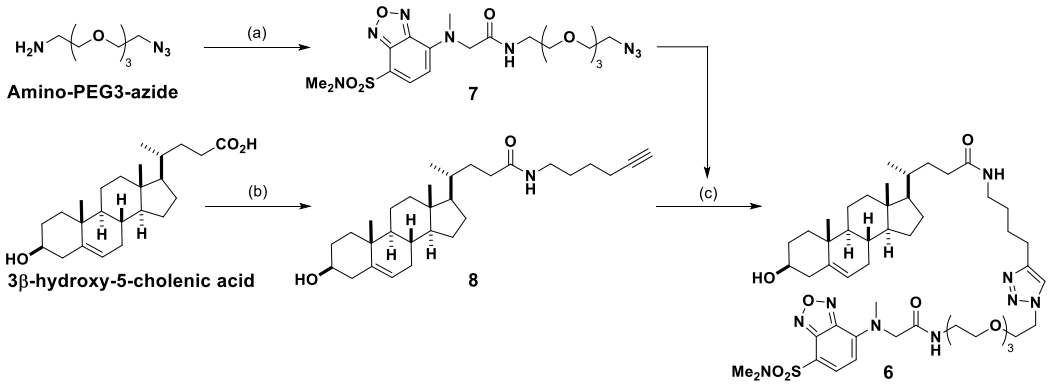


Reagents and conditions: (a) DBD-COCl (2.0 equiv.), Me_2SnCl_2 (2.0 equiv.), collidine (8.0 equiv.), THF (0.1 M), rt, 2h, 48%.

To a solution of compound **1** (2.81 mg, 4.03 μmol) in THF (40 μL) were added Me_2SnCl_2 (1.77 mg, 8.05 μmol), collidine (7.22 mg, 64.4 μmol) and DBD-COCl (2.69 mg, 8.05 μmol), which was stirred for 2 h at room temperature. The reaction mixture was diluted in EtOAc (5 mL) and washed with 3% HCl, water, saturated $NaHCO_3$ aq. solution and brine. The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was purified by flash column chromatography ($CHCl_3$/MeOH = 97/3) to give compound **5** (1.92 mg, 1.93 μmol, 48%) as yellow solid: ^{1}H NMR (500 MHz, pyridine-d_5): 8.04 (1H, d, J = 8.0 Hz), 6.25 (1H, d, J = 8.6 Hz), 5.87-5.84 (1H, m), 5.38 (1H, br),

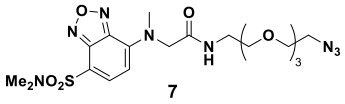
5.33-5.28 (1H, m), 5.12-4.91 (2H, m), 4.86 (1H, s), 4.63 (1H, d, *J* = 6.9 Hz), 4.40 (1H, s), 4.28-4.16 (5H, m), 3.82-3.73 (2H, m), 3.63-3.59 (2H, m), 3.33-3.28 (1H, m), 3.28 (3H, s), 2.83 (6H, s), 2.83-2.79 (2H, m), 2.61 (2H, d, *J* = 7.45 Hz), 2.39 (3H, s), 2.39-2.33 (1H, m), 2.13-2.02 (2H, m), 2.00-1.88 (3H, m), 1.86-1.76 (2H, m), 1.72-1.43 (8H, m), 1.31 (3H, d, *J* = 7.5 Hz), 1.12-1.07 (1H, br), 1.07 (3H, s), 0.96-0.94 (1H, br), 0.95 (3H, s), 0.93 (3H, s), 0.91 (3H, s) ; ^{13}C NMR (125 MHz, pyridine-d_5): δ 219.5, 169.7, 147.7, 145.4, 142.9, 141.9, 139.3, 121.1, 109.6, 106.6, 103.2, 88.1, 86.1, 83.4, 75.3, 74.6, 73.8, 71.8, 71.3, 70.8, 69.0, 67.3, 63.1, 56.5, 50.2, 48.7, 46.5, 46.2, 43.5, 41.2, 39.4, 37.8 (×2), 36.8, 36.2, 32.7, 32.6, 32.5, 32.3, 32.1, 30.0, 27.9, 23.0, 22.6, 21.0, 19.6, 13.7, 12.1; HRMS (ESI-TOF) calcd for $C_{48}H_{72}N_4NaO_{16}S$ (M+Na$^+$): 1015.45617; found: 1015.45611.

3. Synthesis of DBD- sterol probe (6)



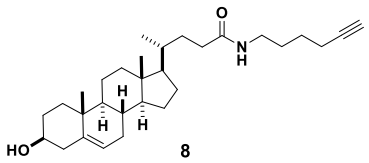
Reagents and conditions: (a) DBD-COCl (1.5 equiv.), DIPEA, CH_2Cl_2, rt, 2 h, 92%; (b) hexynoyl amine (1.1 equiv.), DMT-MM (1.1 equiv.), TEA (6.5 equiv.), DMF, rt, 12 h, 69 %; (c) **7**, $CuSO_4·5H_2O$ (0.1 equiv.), TBTA(0.1 equiv.), disodium ascorbate (0.2 equiv.), *t*BuOH/H_2O = 1/1, 65 °C, 2 h, 73%.

DBD-PEG3-azide (7)



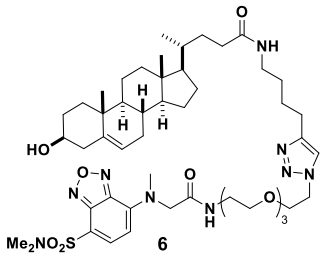
To a solution of 1-amino-11-azido-3,6,9-trioxaundecane (amino-PEG3-azide, 5 mg, 22.9 μmol) in CH_2Cl_2 (91 μL) was added DIPEA (2 equiv) and DBD-COCl (1.0 equiv.), which was stirred at room temperature. Another portion of DBD-COCl (0.5 equiv) was added after 1 h. After 1 h, the mixture was diluted with CH_2Cl_2 and was washed with 3% aqueous HCl, water, saturated $NaHCO_3$ aq. solution and brine. The residue was purified by flash column chromatography. (MeOH/CH_3Cl_2 = 3/97) to give compound **7** (10.85 mg, 21.1 μmol, 92%) as yellow solid: ^{1}H NMR (300 MHz, $CDCl_3$): *δ* 7.88 (1H, d, *J* = 8.3 Hz), 6.63 (1H, s), 6.19 (1H, d, *J* = 8.3 Hz), 4.68 (2H, s), 3.69-3.37 (19H, m), 2.87 (6H, s); ^{13}C NMR(100 MHz, $CDCl_3$): 168.1, 147.1, 144.9, 142.8, 138.7, 110.1, 103.1, 70.7, 70.6, 70.5, 70.4, 70.1, 69.7, 58.0, 50.8, 41.7, 39.5, 37.9 (×2); HRMS (ESI-TOF) calcd for $C_{19}H_{30}NaO_7S$ (M+Na$^+$):537.1856 ; found: 537.1862.

Compound 8



To a solution of aminohexyl hydrochloride[1] (20.0 mg, 0.150 mmol) in THF (1.89 mL) was added 3ß-hydroxy-Δ5-cholenic acid (49.4 mg, 0.132 mmol), TEA (120 µL, 0.861 mmol) and DMT-MM (41.5 mg, 0.150 mmol) dissolved in THF (2.83 mL) and stirred at room temperature for 12h. The reaction mixture was diluted with EtOAc (140 mL), then washed with 10 % citric acid, saturated sodium bicarbonate and brine. The organic layer was dried over Na_2SO_4. The residue was purified by flash column chromatography ($MeOH/CH_2Cl_2$ = 1/99 to 3/97) to give compound **5** (34.1 mg, 0.0752 mmol, 69%) as white solid : 1H NMR (400 MHz, $CDCl_3$): δ 5.51 (m, 1H), 5.33 (d, J = 5.2 Hz, 1H), 3.50 (m, 1H), 3.27 (m, 2H), 2.28 (m, 1H), 2.24 (m, 1H), 2.22 (m, 2H), 2.20 (m, 4H), 1.98 (m, 1H), 1.95 (m, 1H), 1.85 (m, 1H), 1.84 (m, 1H), 1.82 (m, 1H), 1.81 (m, 1H), 1.58 (m, 2H), 1.55 (m, 1H), 1.51 (m, 1H), 1.47 (m, 2H), 1.45 (m, 1H), 1.42 (m, 1H), 1.27 (m, 1H), 1.25 (m, 1H), 1.12 (m, 1H), 1.05 (m, 2H), 0.98 (m, 2H), 0.97 (s, 3H), 0.93 (m, 1H), 0.91 (m, 1H), 0.90 (m, 1H), 0.65 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): HRMS (ESI-TOF) calcd for $C_{30}H_{47}NNaO_2$ ($M+Na^+$) 476.3504; found 476.3502.

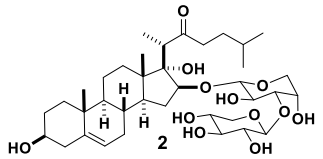
DBD-sterol probe (6)



To a solution of compound **8** (4.39 mg, 10 µmol) in 800 µL of *t*BuOH/ H_2O (1:1) was added 50 µL of DBD-PEG3-azide (**7**) (10.85 mg, 21 µmol) in *t*BuOH (100 µL), 50 µL of a solution of tris[(1-benzyl-1*H*-1, 2, 3-triazol-4-yl)methyl]amine (TBTA, 1.06 mg, 2.0 µmol) in *t*BuOH (100 µL), 50 µLof a solution of disodium 4 ascorbate (3.96 mg, 27 µmol) in water (500 µL), and 50 µL of a solution of $CuSO_4 \cdot 5H_2O$ (2.5 mg, 10 µmol) in water (500 µL). The reaction mixture was stirred at 65 °C for 2 h. The Quadra pure™ (Sigma-Aldrich) was added to the reaction mixture, which was stirred for 2 h. The resin was filtered and the filterate was concentrated in vacuo. The residue was purified by flash column chromatography ($MeOH/CH_2Cl_3$ = 5/95) to give compound **6** (7.04 mg, 7.3 µmol, 73%) as yellow solid: ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.85 (1H, d, J = 8.3 Hz,), 7.45 (1H, s), 6.18 (1H, d, J = 8.6 Hz), 5.85 (1H, t, J = 5.3 Hz), 5.32 (1H, d, J = 4.8 Hz), 4.69 (2H, s), 4.48 (2H, t, J = 5.0 Hz), 3.86 (2H, t, J = 5.0 Hz), 3.70-3.37 (19H, m), 3.22 (2H, q, J = 12.9 Hz), 2.84 (6H, s), 2.70-2.61 (2H, m), 2.31-2.16 (4H, m), 2.08-1.92 (3H, m), 1.82 (3H, d, J = 10.3 Hz), 1.66-1.61 (2H, m), 1.56-1.20 (9H, m), 1.13-0.89 (13H, m), 0.64 (3H, s); ^{13}C NMR (75 MHz, $CDCl_3$): 173.9, 168.3, 147.7, 147.1, 145.0, 142.9, 140.9, 138.8, 122.1, 121.7, 109.6, 102.8, 71.8, 70.6, 70.5, 70.3, 69.7,

69.6, 57.9, 56.8, 55.9, 50.1, 42.4, 41.8, 39.8 (×2), 39.4, 39.2, 37.9, 37.3, 36.6, 35.6, 33.7, 31.9, 31.7, 29.1, 28.3, 26.8, 25.2, 24.3, 21.1, 19.5, 18.5, 12.0; HRMS (ESI-TOF) calcd for $C_{49}H_{77}NaO_9S$ ($M+Na^+$): 990.5463 ; found:990.5446.

4. Isolation of deacetylated OSW-1 (2) from *Ornithogalum saundersiae*



2

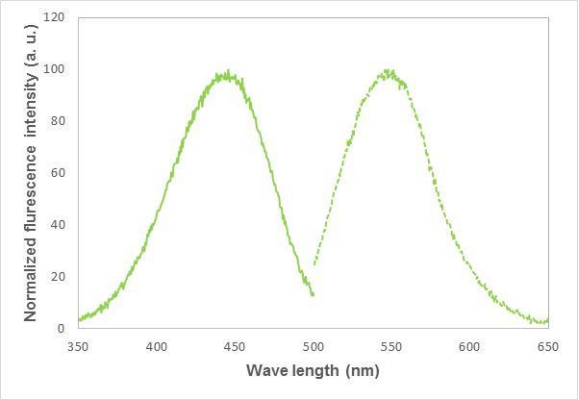
Deacetylated OSW-1 (**2**) was isolated from the roots of *Ornithogalum saundersiae* based on the previously published protocol.[2] The fresh roots were diced and lyophilized (204 g) which were washed with hexane then were refluxed with methanol (400 mL) for 1 h. After cooling to ambient temperature, the pulps were filtered. The filtered residues were subjected to two additional rounds of methanolic extraction. The combined methanolic extract was concentrated *in vacuo* and was diluted in 80 % MeOH/H_2O solution. The aqueous methanol layer was washed with hexane (× 2) and CH_2Cl_2 (× 3). The CH_2Cl_2 layer was collected and was reduced in volume *in vacuo*, which was washed with brine (× 2). The CH_2Cl_2 layer was dried over Na_2SO_4 and was concentrated *in vacuo*. The crude fraction (8 g) was purified by silica gel chromatography (0%→2%→3%→4%→5%→10%→20%→50% MeOH/$CHCl_3$) twice and the fraction eluted with 5% MeOH/$CHCl_3$ was collected at each round. The crude sample was then purified by ODS chromatography on Biotage Isolera One system (25−50% MeCN/H_2O gradient) to collect the fraction eluted 36−39% MeCN/H_2O gradient. The collected fraction was further purified by an analytical scale reversed-phase HPLC using a 4.6 × 25.0 mm (5 μm) Waters Xbridge ODS column at a flow rate of 1 mL/ min (45−51% MeCN/H_2O gradient) with UV detection at 192 nm to give compounds **2** (9 mg): ^{1}H NMR (500 MHz, pyridine-d_5): 5.85 (1H, t, *J* = 7.0 Hz), 5.38 (1H, d, *J* = 4.0 Hz), 4.90 (1H, d, *J* = 7.5 Hz), 4.86 (1H, s), 4.63 (1H, d, *J* = 6.0 Hz), 4.45 (1H, m), 4.33 (1H, dd, *J* = 14.5 Hz, *J* = 8.0 Hz), 4.27-4.20 (3H, m), 4.14 (1H, m), 4.08 (1H, t, *J* = 8.5 Hz), 3.86-3.76 (3H, m), 3.69 (1H, t, *J* = 10.5 Hz), 3.32 (1H, dd, *J* = 7.5 Hz, *J* = 14.5 Hz), 2.82 (2H, t, *J* = 7.5 Hz), 2.61 (2H, d, *J* = 7.5 Hz), 2.38 (1H, m,), 2.36 (3H, s), 2.13-2.04 (2H, m), 2.00-1.87 (2H, m), 1.84-1.76 (2H, m), 1.73-1.40 (8H, m), 1.31 (3H, d, *J* = 7.5 Hz), 1.24 (1H, br), 1.11 (1H, m), 1.07 (3H, s), 0.98 (1H, br), 0.95 (3H, s), 0.93 (3H, d, *J* = 6.0 Hz), 0.91 (3H, d, *J* = 6.5 Hz); ^{13}C NMR (125 MHz, pyridine-d_5): δ 219.0, 170.0, 141.9, 121.1, 106.9, 101.4, 88.2, 85.7, 80.2, 78.3, 74.2, 72.2, 71.3, 70.9, 68.8, 67.2, 66.7, 50.1, 48.6, 46.5, 46.4, 43.5, 39.5, 37.7, 36.8, 35.1, 32.8, 32.7, 32.6, 32.2, 32.0, 27.9, 22.8, 22.5, 21.5, 20.9, 19.6, 13.5, 11.9; HRMS (ESI-TOF) calcd for $C_{39}H_{62}NaO_{13}$ ($M+Na^+$):761.40881; found: 761.40605.

5. Fluorescence spectroscopic analysis of DBD-tagged probes

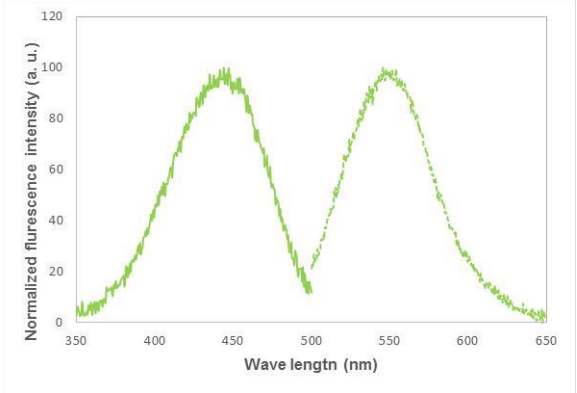
Fluorescence spectra were recorded for **3-6** as 1 μM solution in PBS. The excitation spectra or emission spectra for an appropriate spectral window were recorded on JASCO ETC 273 spectrophotometer (JASCO) using the excitation scan or emission scan mode. Fluorescence excitation spectra were recorded with an emission at 555 nm and emission spectra were recorded with an excitation at 445 nm. Fluorescence excitation/emission spectra for probe **3** has been

reported previously.[2]

(a)



(b)



(c)

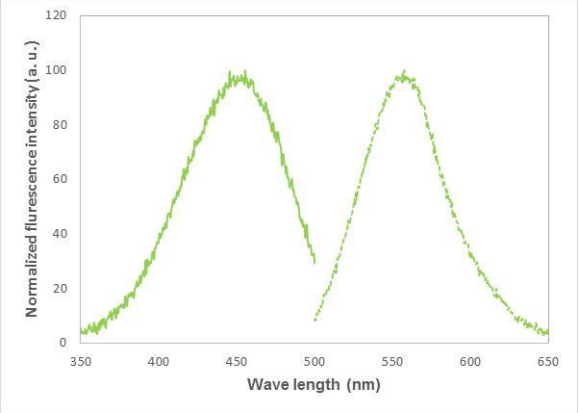


Figure S1. Fluorescence excitation (single line) and emission spectra (dashed line) of (a) probe **4**, (b) probe **5**, (c) probe **6** at 1 μM in PBS.

6. Cell cultures

HeLa cells (RIKEN bioresource center) were cultured in DMEM (Gibco) supplemented with 10% FBS 100 U/mL penicillin and 100 μg/mL streptomycin sulfate in a humidified 5% CO_2 atmosphere at 37 °C.

7. XTT Assay

HeLa cells were trypsinized and suspended in culture media (DMEM supplemented with 10% FBS 100 U/mL penicillin and 100 μg/mL streptomycin sulfate) at a final concentration of 1×10^4 cells/mL, which were seeded in a 96-well microtiter plate. Varied concentrations of compounds (OSW-1, **1**-**5**)[3] or DMSO were added to each well in triplicate, and then were incubated for 72 h. They were incubated with 2 mg/mL XTT (Sigma-Aldrich) in culture media for 4 h. The amounts of viable cells were determined by measuring the UV absorbance at 490 nm on Micro Plate Reader Model 550 (Biorad). The IC_{50} values were obtained by non-linear regression curve-fitting to a Hill's equation using the Prism program (GraphPad).

8. Cell internalization studies

HeLa cells were seeded in 96-well plates at a density of 1×10^4 cells/well in 100 μL media, which were then treated with 1 μM DBD-tagged probes at 37 °C for the indicated time, and washed 3 times with 300 μL PBS buffer. Cells were then lysed with 0.1 μL of RIPA lysis buffer (1.37 mM NaCl, 0.268 mM KCl, 2.5 mM Tris-HCl, 0.01% Triton X-100, 0.1% SDS, 1.2 mM sodium deoxycholate, a tablet/25 mL of protease inhibitor cocktail (Roche Complete Mini, EDTA free)) for 30 min at room temperature. The fluorescence signal from each well was measured (ex. 450 nm, em. 560 nm) by Corona MICROPLATE READER SH-9000 lab (Hitachi). Averaged data ± SD for triplicate experiments for each time point were calculated using Excel.

9. Fluorescence cell imaging studies

HeLa cells were seeded in a glass-bottom dish (IWAKI) at 3×10^5 cells/mL in DMEM and were cultured for 20 h. For mitochondria staining, cells were treated with DBD-tagged probes **3-5** (1 μM) at 37 °C for 1 h and with tetramethyl rhodamine ethyl ester (TMRE, 25 nM) for further 10 min and then were imaged live. For ER staining, cells were preincubated with 0.25 μM ER-Tracker™ Red (BODIPY® TR-glibenclamide, Thermo Fisher Scientific) in HBSS buffer (0.34 mM Na_2HPO_4, 0.44 mM $KHPO_4$, pH 7.2, 137.9 mM NaCl, 1.26 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$, 5.33 mM KCl, 4.17 mM $NaHCO_3$;Gibco) at 37 °C for 1 h then treated with DBD-probe **3-5** (1 μM) at 37 °C for 1.5 h for **2** or 1 h for **7**, washed with chilled PBS, fixed with 4% paraformaldehyde and were analyzed. For Golgi staining, cells were preincubated with 3 μM BODIPY® TR-ceramide (Thermo Fisher Scientific) in HBSS buffer at 17 °C for 30 min, washed with HBSS buffer then were incubated with DBD-probe **3-5** (1 μM) at 37 °C for 30 min and after washing with chilled PBS were imaged live. For lysosome staining, cells were preincubated with 100 nM Lysotracker® Red (Thermo Fisher Scientific) in DMEM at 37 °C for 1 h, washed with DMEM, then treated with DBD-probe **3-5** (1 μM) at 37 °C for 1.5 h, washed with chilled PBS, fixed with 4% paraformaldehyde and were analyzed. For fluorescence microscopic imaging, the glass-bottom dish was placed on the stage of an inverted epifluorescence microscope (IX-70; Olympus) equipped with a 40×objective lens (Uapo 40×3/340, NA =0.9; Olympus). Fluorescence was elicited by illumination with a 75 W xenonlamp through a 15nm band-pass filter centered at 535nm. Fluorescence at >580nm was collected with a cooled CCD camera (Sensicam QE (6.45 μm /pixel at 1×), PCO AG, Kelheim, Germany). For DBD fluorescence, excitation between 450 and 480nm and emission between 515 and 550 nm were used. The images were acquired with binning pixels 2×2. The exposure time for each frame was 1 s. The intensity of illumination was also reduced to 25% with a neutral density filter to avoid photodynamic injury to cells. All procedures described above were performed at room temperature. The image data with the resolution of 12 bits per pixel were analyzed with Image J. Pearson's correlation coefficient were calculated to evaluate the correlation between the localization of DBD-probes (**3**-**5**) and that of ER, Golgi or mitochondria marker using image-processing software (Meta-Morph; MolecularDevices Inc.,Sunnyvale,CA). The average data and standard deviation for Pearson's correlation coefficient were derived for at least 4 cells per sample (Table S1).

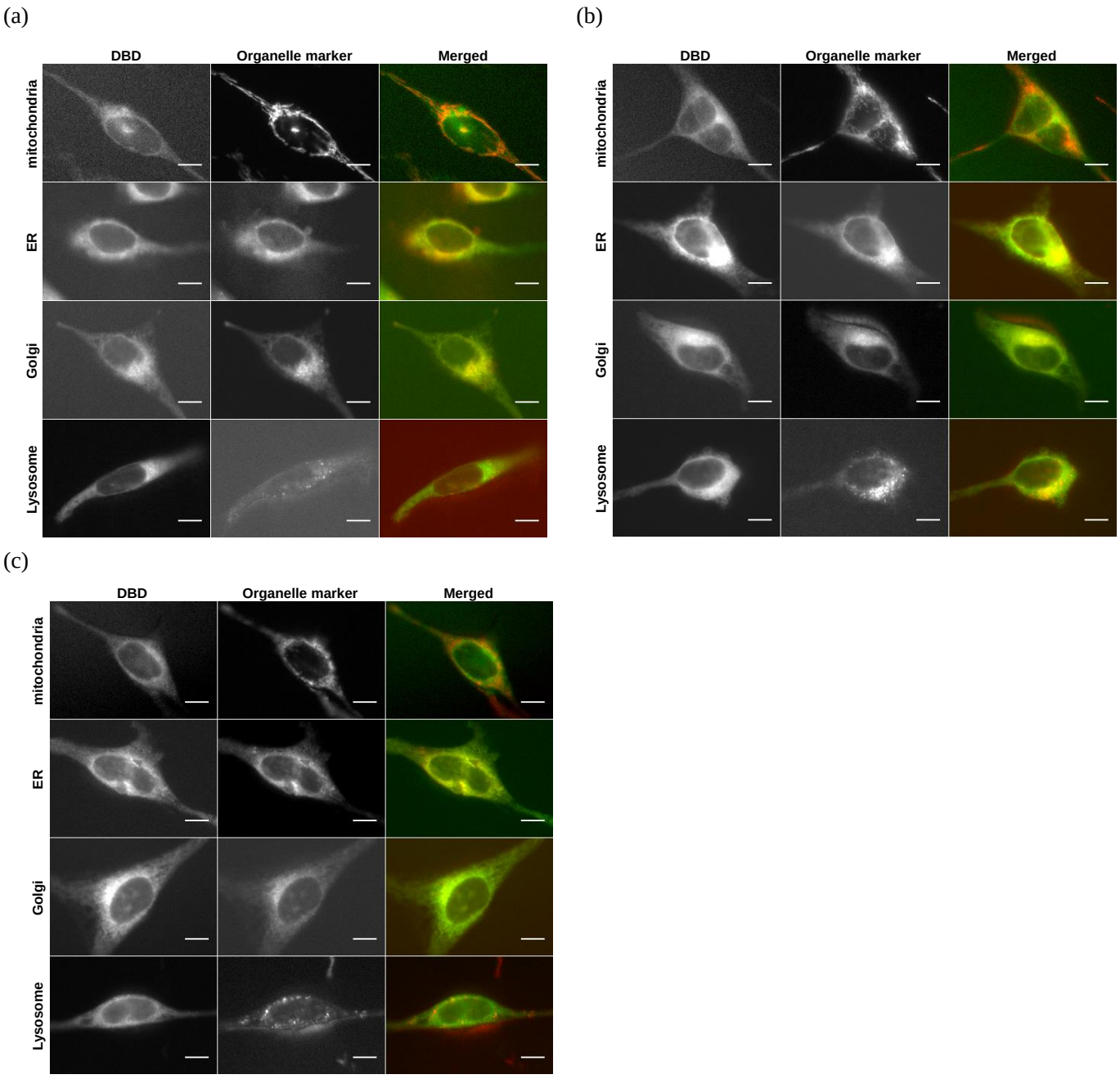


Figure S2. Intracellular localization analysis of (a) probe **3**, (b) probe **4**, and (c) probe **5** in HeLa cells by fluorescent microscopy. Cells were treated with DBD-probes (1 μM) and tetramethylrhodamine ethylester as a mitochondria marker, ER tracker as an ER marker or BODIPY TR-ceramide as a Golgi marker or Lysotracker Red as a lysosome marker. In merged images, fluorescence of organelle specific stains are shown in red and that of DBD are shown in green. Scale bars: 10 μm.

	Probe 3	**Probe 4**	**Probe 5**
Co-localization with ER marker	0.8415 ± 0.0456	0.9514 ± 0.0313	0.9425 ± 0.0274
Co-localization with Golgi marker	0.9375 ± 0.0460	0.9716 ± 0.0085	0.9428 ± 0.0310
Co-localization with mitochondria marker	0.5384 ± 0.1457	0.6385 ± 0.1035	0.5437 ± 0.1145

Table S1. Co-localization efficiency for probe **3-5** and ER, Golgi and mitochondria marker by Pearson's correlation coefficient.

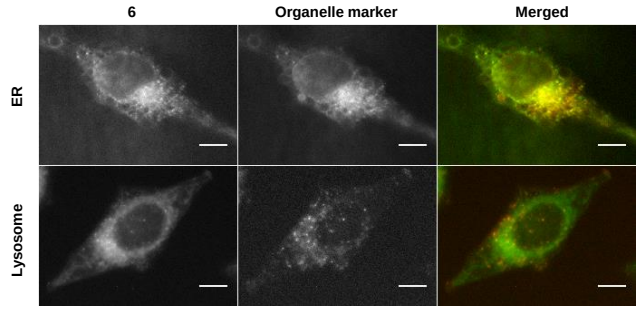


Figure S3. Fluorescence imaging analysis of HeLa cells treated with DBD-tagged sterol probe **6**. Cells were treated with **6** (1 μM) and ER tracker as an ER marker Lysotracker Red as a lysosome marker. In merged images, fluorescence of organelle specific stains are shown in red and that of DBD are shown in green. Scale bars: 10 μm.

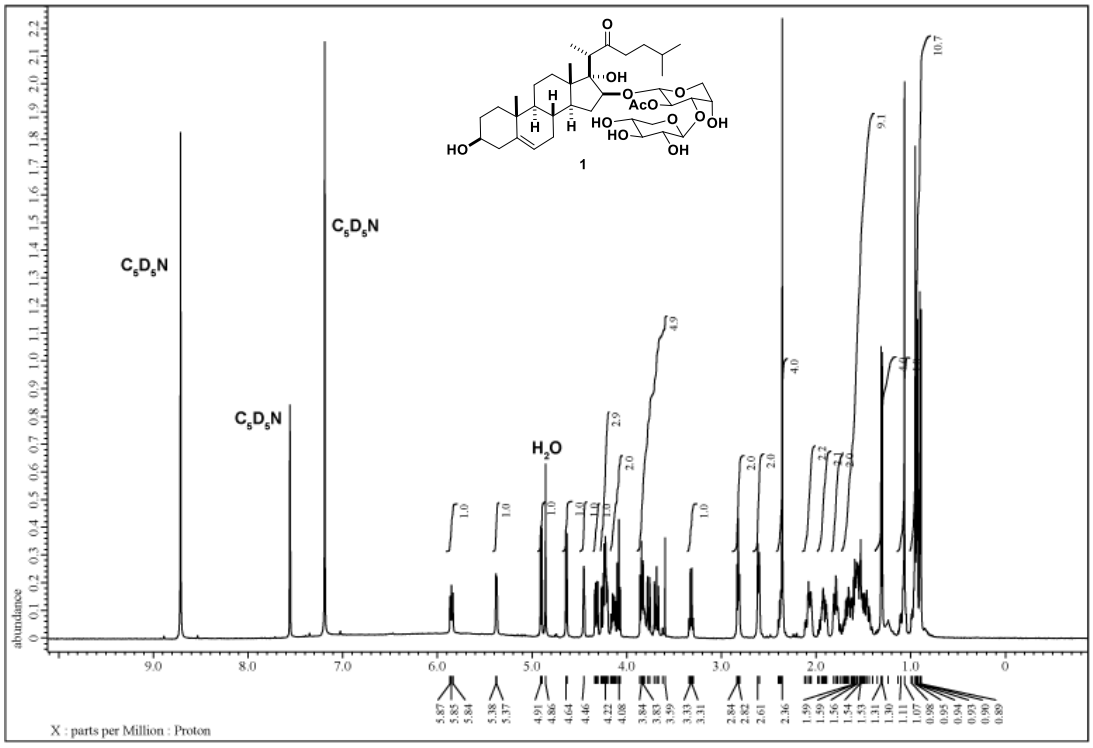
References

1. B. L. Nilsson, L. E. Overman, J. R. de Alaniz, J. M. Rohde. *J. Am. Chem. Soc.*, **2008**, *130*, 11297.

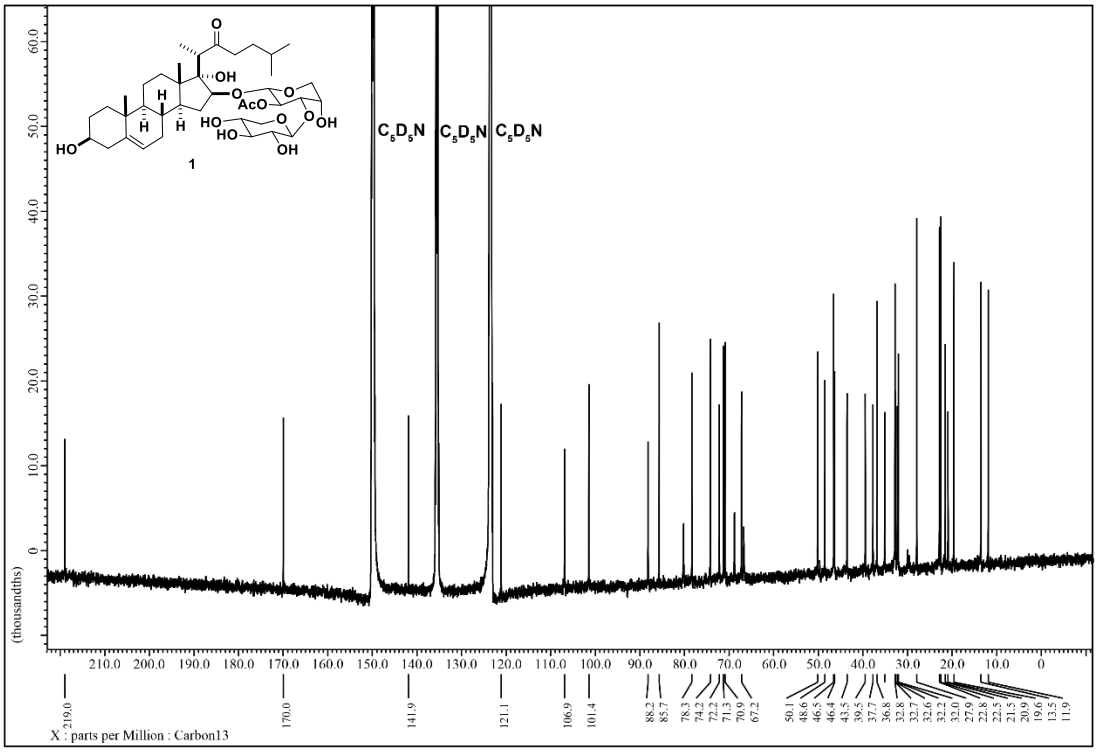
2. K. Sakurai, T. Takeshita, M. Hiraizumi, R. Yamada, *Org. Lett.* 2014, **16**, 6318.

3. K. Sakurai, T. Fukumoto, K. Noguchi, N Sato, H. Asaka, N. Moriyama M. Yohda., *Org. Lett.* 2010, **12**, 5732.

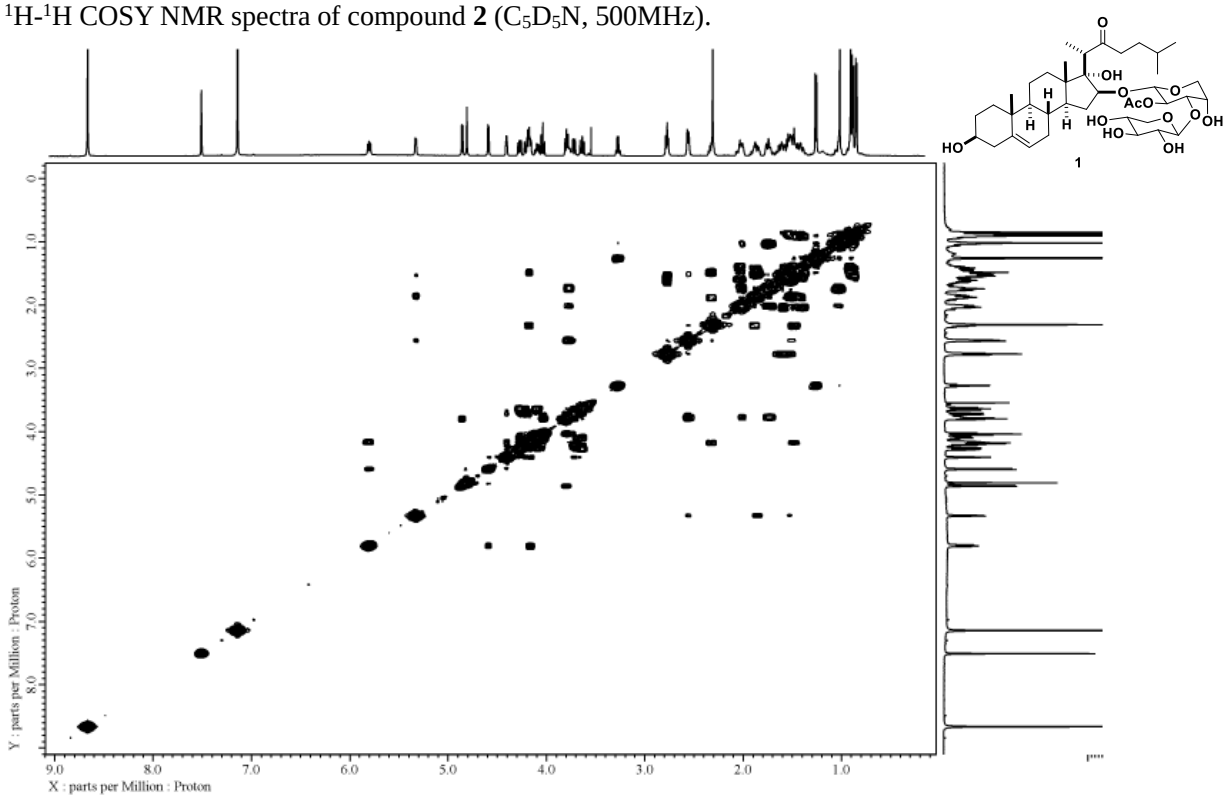
^{1}H NMR spectra of compound **2** (C_5D_5N, 300MHz).



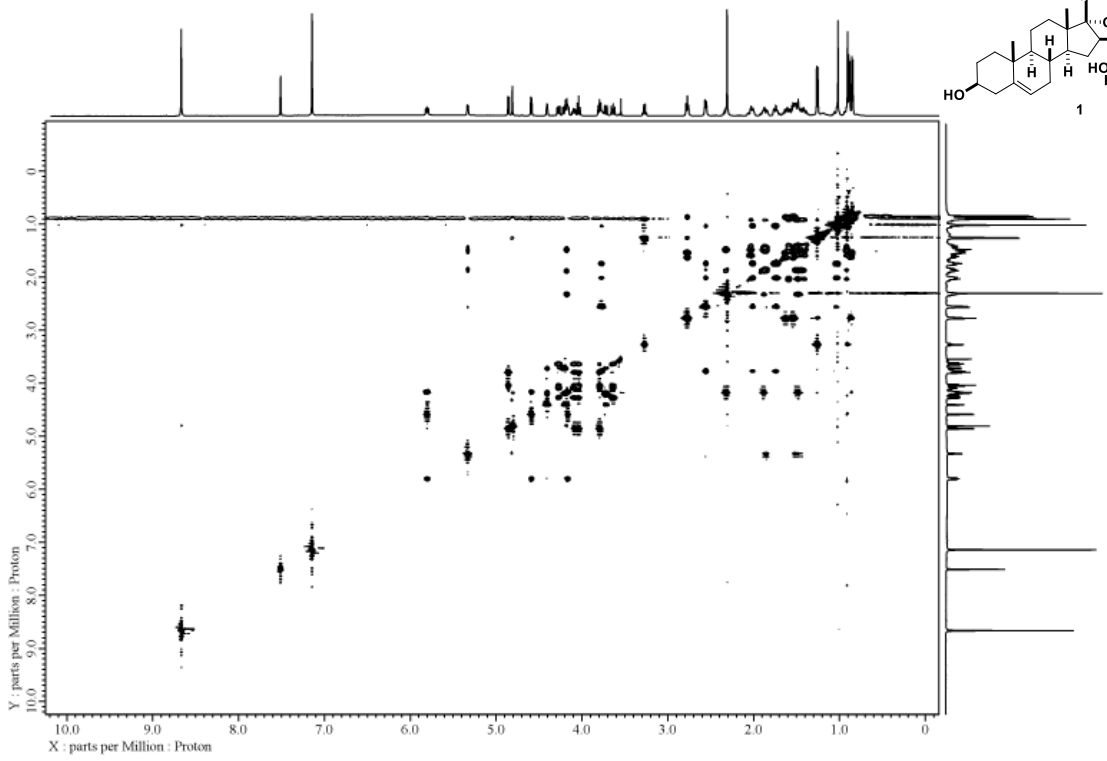
^{13}C NMR spectra of compound **1** (C_5D_5N, 75MHz).

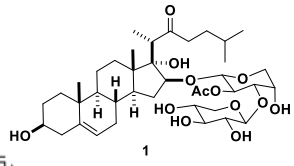


^{1}H-^{1}H COSY NMR spectra of compound **2** (C_5D_5N, 500MHz).

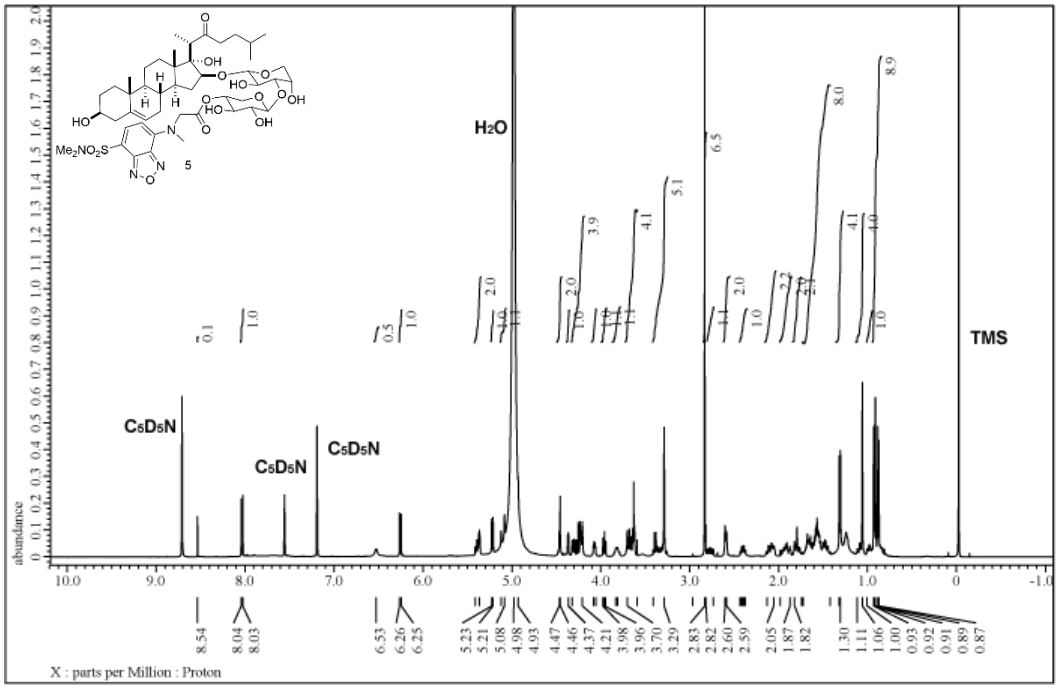


^{1}H-^{1}H TOCSY NMR spectra of compound **2** (C_5D_5N, 500MHz).

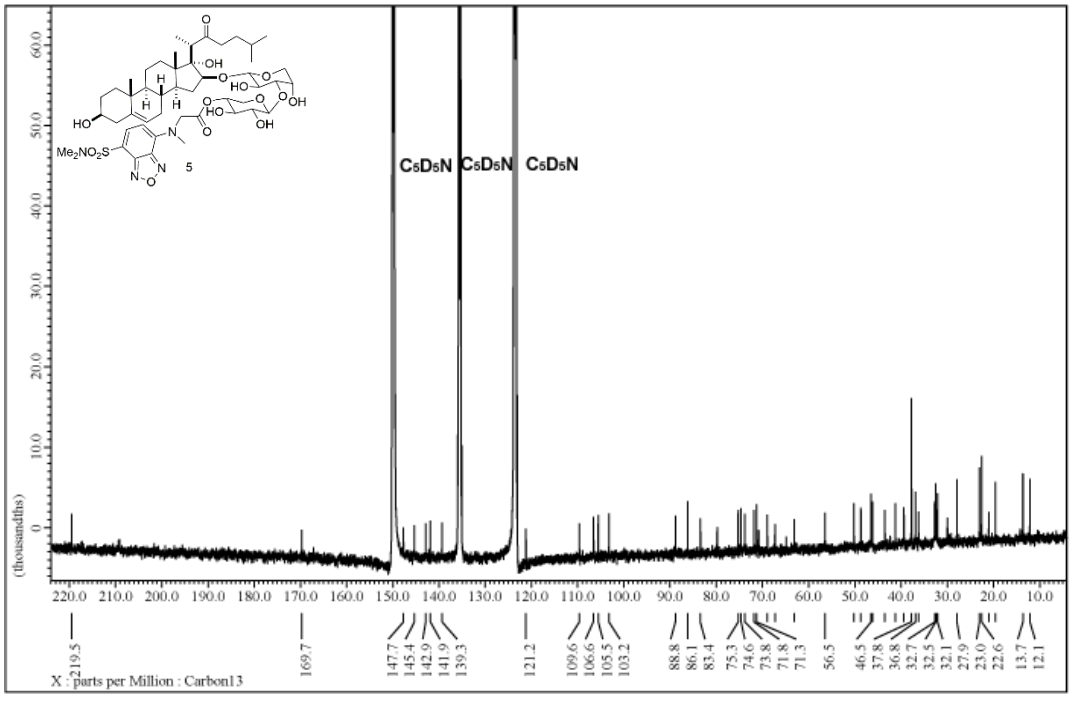




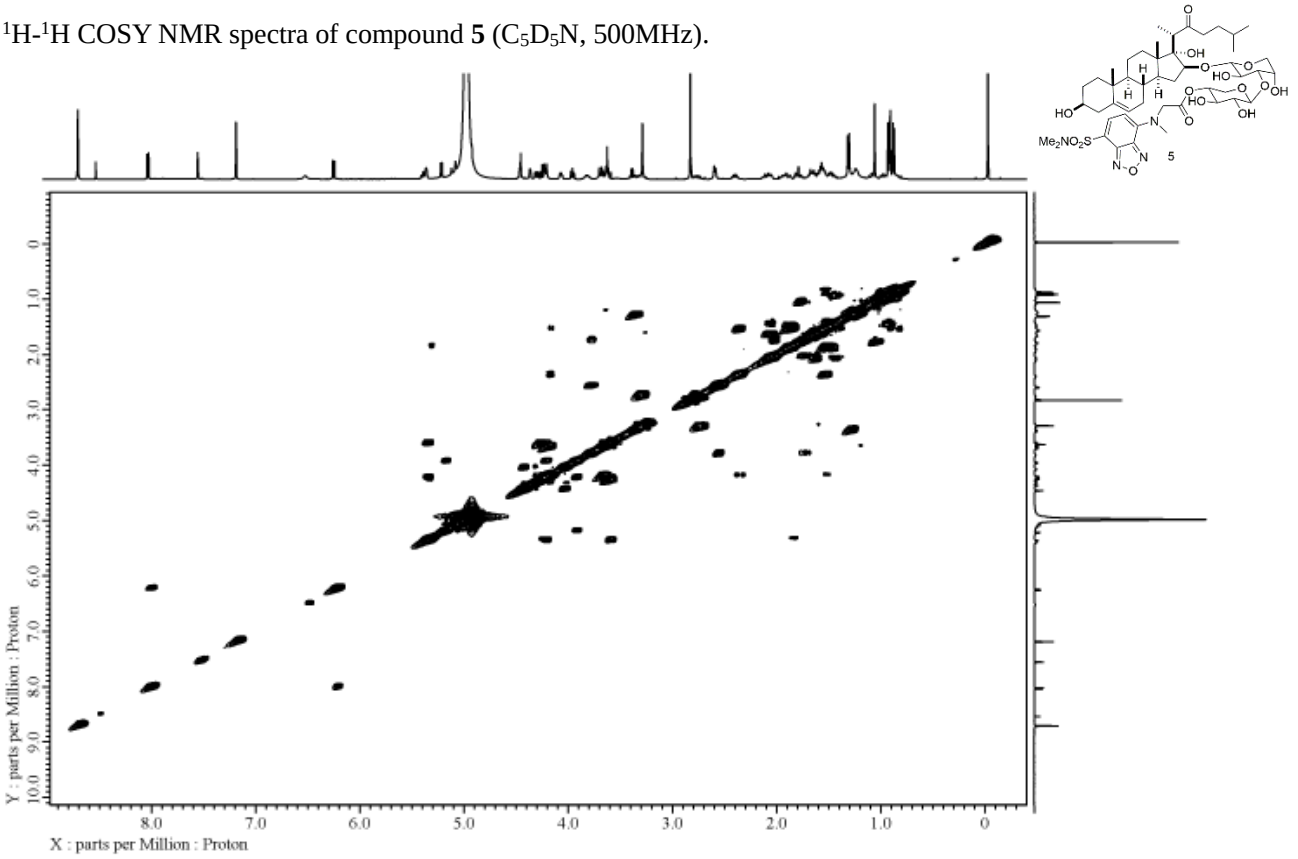
^{1}H NMR spectra of compound **5** ($CDCl_3$, 300MHz).



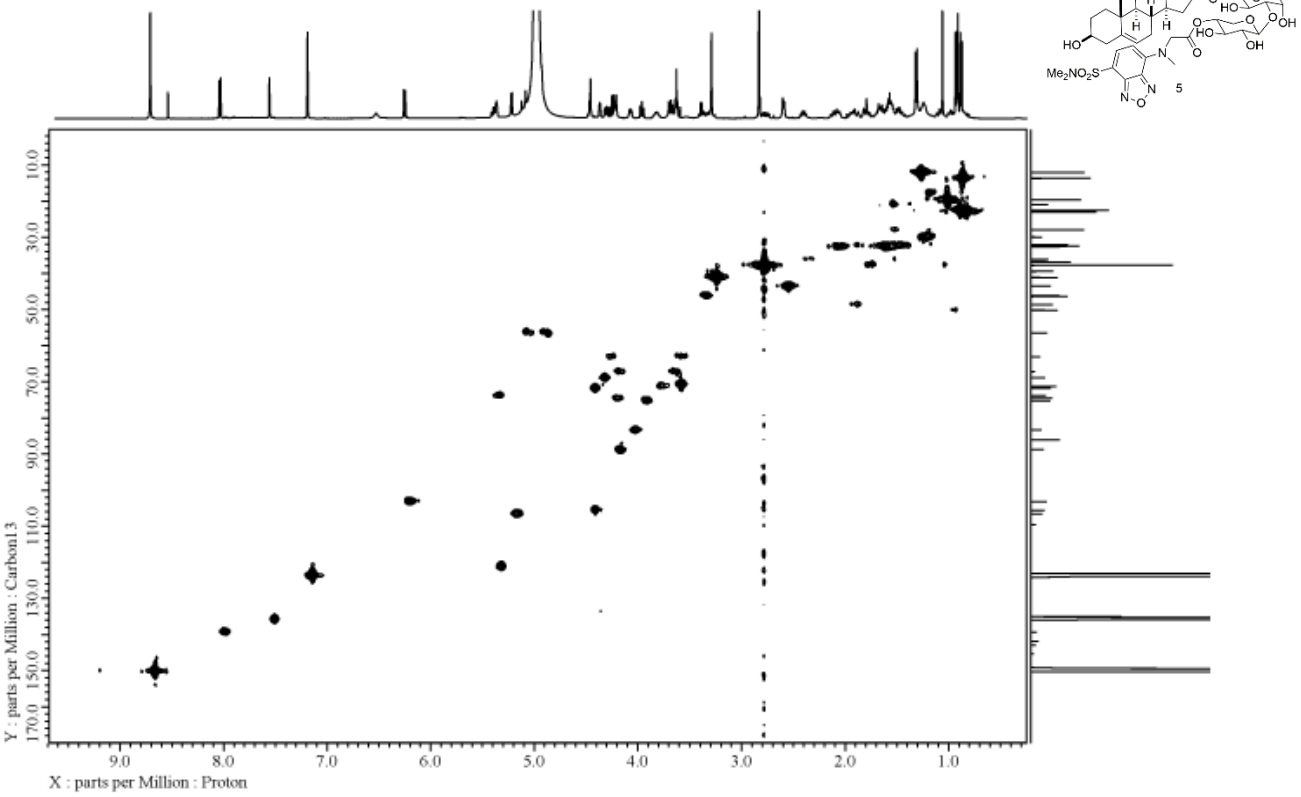
^{13}C NMR spectra of compound **5** (C_5D_5N, 75MHz).



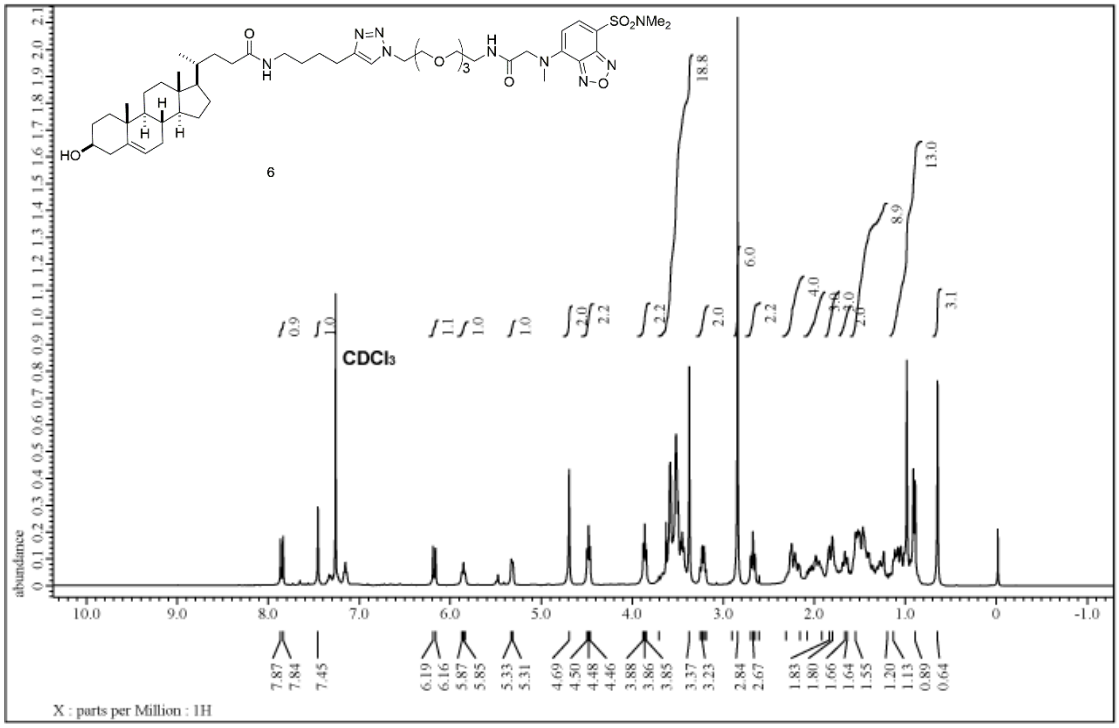
^{1}H-^{1}H COSY NMR spectra of compound **5** (C_5D_5N, 500MHz).



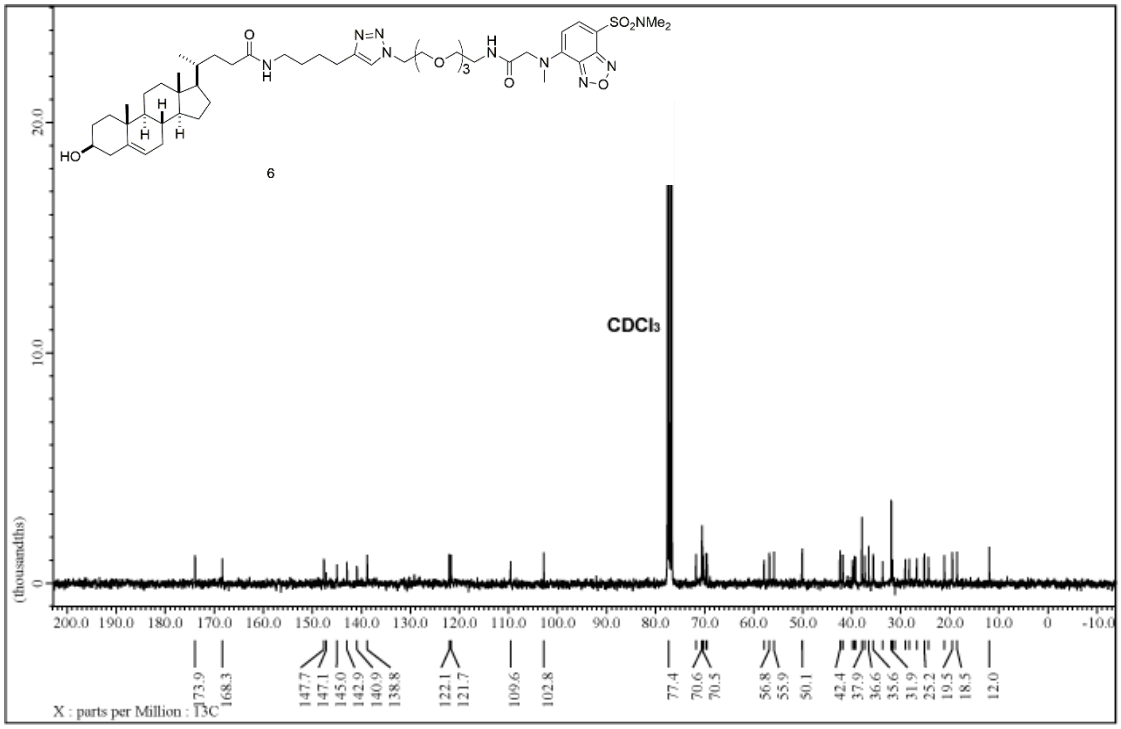
^{1}H- ^{13}C HMQC NMR spectra of compound 1 (C5D5N, 500MHz)



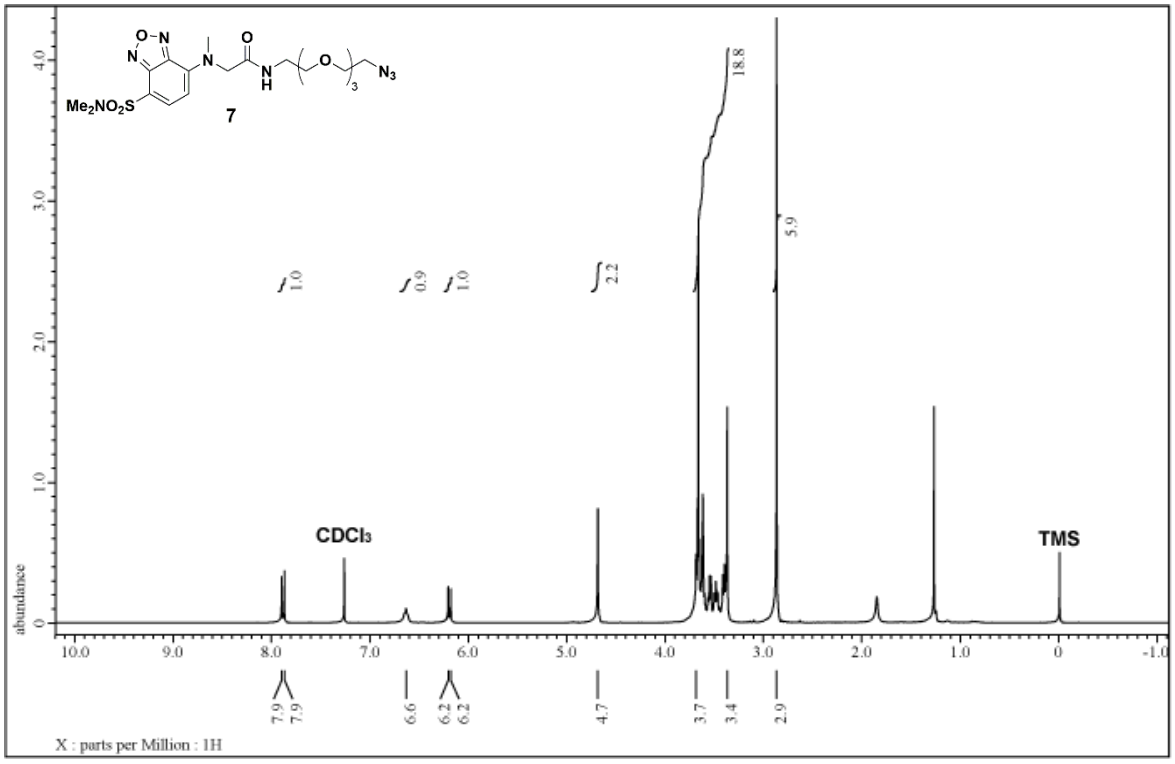
^{1}H NMR spectra of compound **6** ($CDCl_3$, 300MHz).



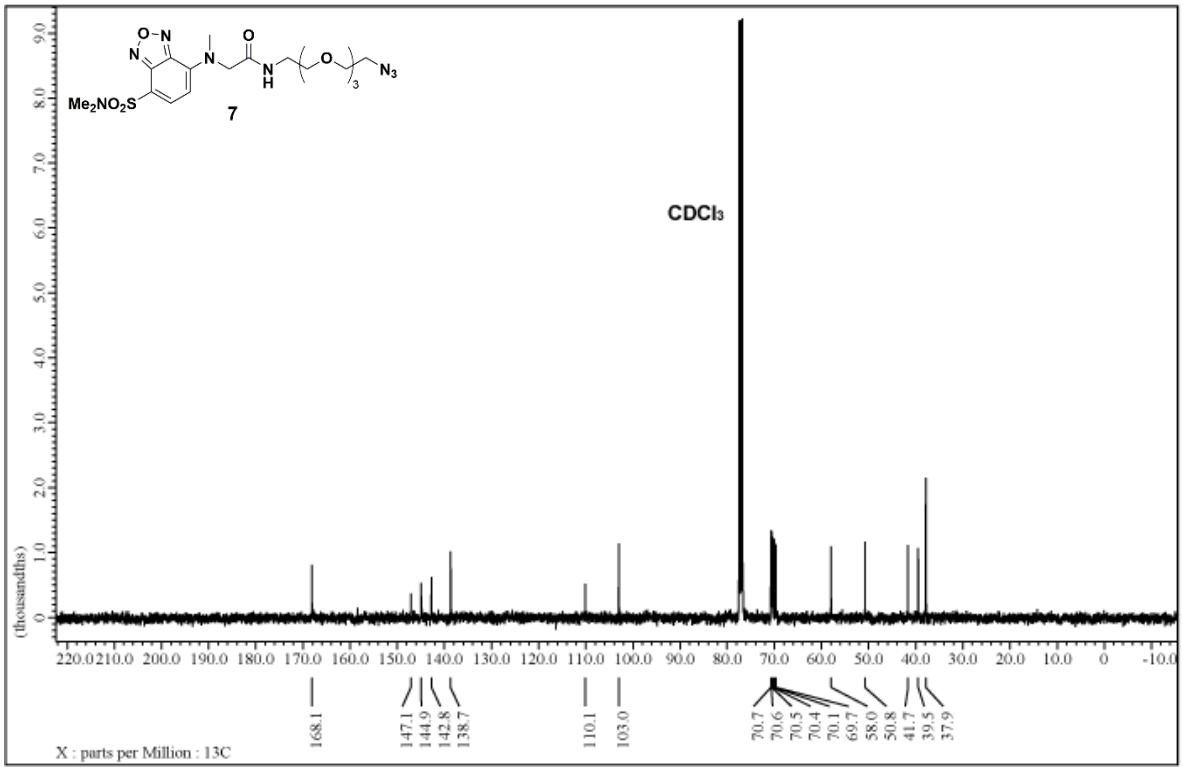
^{13}C NMR spectra of compound **6** ($CDCl_3$, 100MHz).



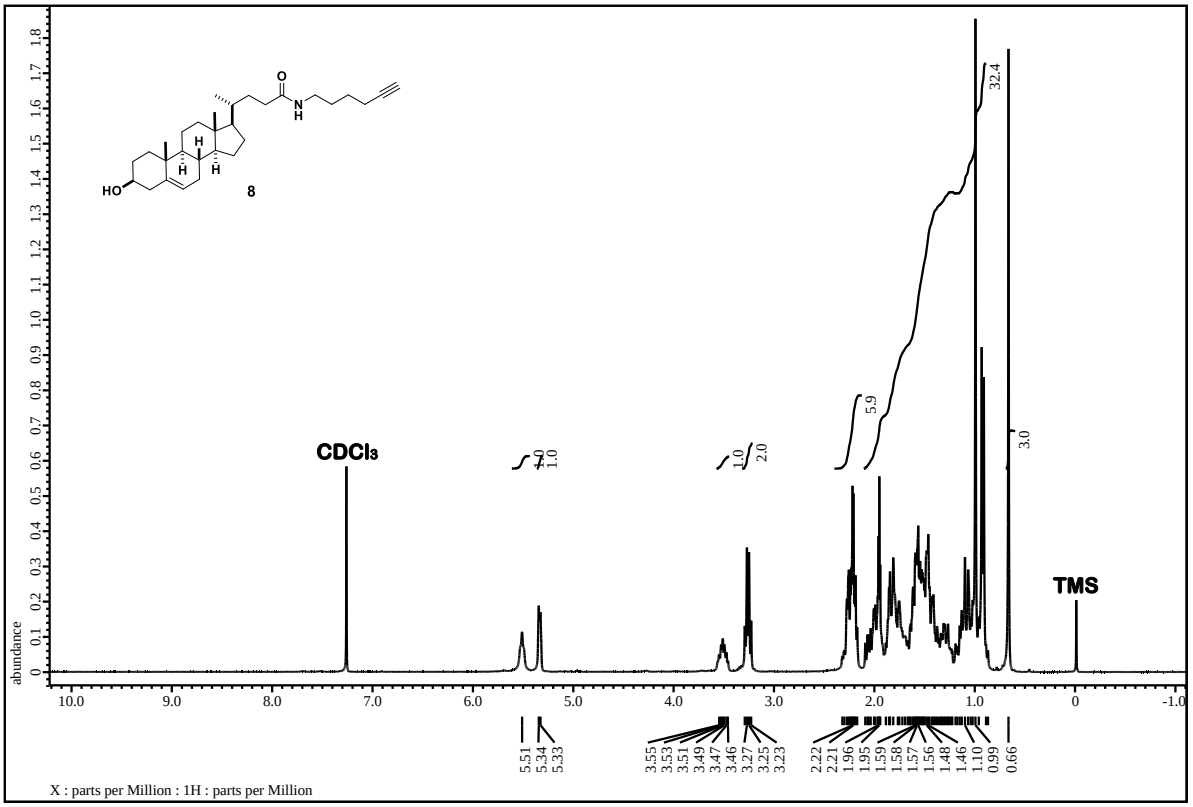
^{1}H NMR spectra of compound **7** ($CDCl_3$, 400MHz).



^{13}C NMR spectra of compound **7** ($CDCl_3$, 100 MHz).



^{1}H NMR spectra of compound **8** ($CDCl_3$, 400MHz).



^{13}C NMR spectra of compound **8** ($CDCl_3$, 100 MHz).