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Supplementary Information

Carrier-free Nano-prodrugs for Minimally Invasive Cancer Therapy

Keita Tanita[1], Yoshitaka Koseki[1,2*], Sanjay Kumar[1], Farsai Taemaitree[1,2], Asuka Mizutani[1], Hirotaka Nakatsuji[1], Ryuju Suzuki[1], Anh Thi Ngoc Dao[3], Fumiyoshi Fujishima[4], Hiroshi Tada[5], Takanori Ishida[5], Ken Saijo[6,7], Chikashi Ishioka[6,7], Hitoshi Kasai[1*]

[1]*Institute of Multidisciplinary Research for Advanced Materials, Tohoku University*

[2]*Research Institute for Electronic Science, Hokkaido University*

[3]*Graduate School of Engineering, Nagasaki University*

[4]*Department of Pathology, Tohoku University Hospital*

[5]*Department of Breast and Endocrine Surgical Oncology, Tohoku University Graduate School of Medicine*

[6]*Department of Medical Oncology, Tohoku University Hospital*

[7]*Department of Clinical Oncology, Tohoku University Graduate School of Medicine*

*Corresponding E-mail address:
koseki@tohoku.ac.jp (Y. Koseki) and kasai@tohoku.ac.jp (H. Kasai).

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1. General Information

Materials: SN-38, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl), caprylic acid and capric acid were purchased from Tokyo Chemical Industry Co. Pyridine, 4-dimethylaminopyridine (DMAP), triethylamine (TEA), tetrahydrofuran (THF), dichloromethane (CH_2Cl_2), acetone, dimethyl sulfoxide (DMSO), ethanol (EtOH), caproic acid, (+/−)-dithiothreitol, trifluoroacetic acid (TFA), acetyl chloride, triphosgene, 1,6-hexanediol, butyric acid, di-tert-butyl decarbonate and phosphate buffered saline (PBS) were purchased from FUJIFILM Wako Pure Chemical Corporation. Chloroform ($CHCl_3$) and methanol (MeOH) were purchased from Nacalai Tesque, Inc. 4,4'-Dithiodibutyric acid, 2-hydroxyethyl disulfide, and mouse serum were purchased from Sigma-Aldrich Co. Acetonitrile, dimethyl sulfoxide-d_6 (DMSO-d_6), chloroform-d ($CDCl_3$), and Silica gel 60N (230–400 mesh) for flash chromatography were purchased from Kanto Chemical co. Silica gel plates 60F254 for thin layer chromatography (TLC), and Whatman® Nuclepore Track-Etched Membrane (ϕ = 0.05 μm) were purchased from Merck. HCT-116 (Human colon cancer) and KPL-4 (Human breast cancer) cells were purchased from RIKEN cell bank. Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), and trypsin were purchased from Life Technologies. Cell counting Kit-8 was purchased from Dojindo Molecular Technologies, Inc. Ketamine Hydrochloride was purchased from Daiichi Sankyo Company, Limited. All the chemicals were used without further purifications.
All chemicals were obtained from commercial sources and were used as received

Instruments: Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVANCE-400 and 500 spectrometers. High-resolution mass spectrometry (HR-MS) was performed using a Bruker micrOTOF-Q II-S1 by electrospray ionization time of flight (ESI-TOF) reflectron experiment. Average particle size, size distributions, and zeta potential were measured using a Malvern Zetasizer nanoZS. Scanning electron microscope (SEM) images were observed using a S-4800 Hitachi. Cell viability was evaluated using a microplate reader (Bio-Rad iMark). High-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) was performed using an HPLC (Agilent 1260 Infinity) connected to a mass spectrometer (Bruker HCT ultra-IMR).

General method of organic synthesis: All air- or moisture-sensitive reactions were carried out in flame-dried glassware under an argon atmosphere. Reactions were monitored by analytical TLC carried out on 0.25-mm silica gel plates. Visualization of the developed plate was performed using UV absorbance at 254 nm or 365 nm. 12 molybdo(VI) phosphoric acid n-hydrate 5% EtOH solution was used as a coloring reagent. Flash chromatography was performed on silica gel 60N (40–50 μm) with the indicated solvent systems. NMR spectra were calibrated using residual undeuterated solvent as an internal reference; $CDCl_3$ at δ = 7.26 ppm for ^{1}H, and δ = 77.16 ppm for ^{13}C NMR. DMSO-d_6 at δ = 2.50 ppm for ^{1}H, and δ = 39.52 ppm for ^{13}C NMR. The following abbreviations were used to explain NMR peak multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, dd = double doublet, m = multiplet, br = broad.

2. Synthesis of SN-38 derivatives

Synthesis of SNC6DE: SN-38×C6 (106.2 mg, 0.216 mmol) and 4,4'-dithiodibutyric acid (27.9 mg, 0.117 mmol) were dissolved in CH_2Cl_2 (2.2 mL). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl) (175.1 mg, 0.913 mmol) and 4-(dimethylamino)pyridine (DMAP) (9.1 mg, 0.075 mmol) were added sequentially at room temperature. The reaction mixture was stirred overnight at 25 °C, followed by it was diluted with CH_2Cl_2, filtered and washed with saturated aqueous solution of NH_4Cl and brine simultaneously. The organic layer were separated, mixed and dried with $MgSO_4$. Concentration *in vacuo* afforded a residue, which was purified by silica gel column chromatography ($CHCl_3$/MeOH = 100/0 → 100/1) to get SNC6DE (87.1 mg, 0.074 mmol, 68%) as a pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 0.96 (6H, t, J = 7.0 Hz), 1.36–1.44 (7H, m), 1.78–1.89 (4H, m), 2.07–2.29 (2H, m), 2.45–2.58 (2H, m), 2.61–2.68 (4H, m), 3.13 (2H, q, J = 7.7 Hz), 5.24 (2H, s), 5.37 (1H, d, J = 17.2 Hz), 5.7 (1H, d, J = 17.2 Hz), 7.12 (1H, s), 7.55 (1H, dd, J = 9.0, 2.8 Hz), 7.82 (1H, d, J = 2.8 Hz), 8.26 (1H, d, J = 9.2 Hz). ^{13}C NMR (100 MHz, $CDCl_3$, δ): 7.67, 14.0, 22.4, 23.2, 23.9, 24.6, 31.4, 31.9, 32.0, 34.5, 37.3, 49.4, 67.2, 76.0, 95.8, 114.7, 120.0, 125.6, 127.4, 127.6, 131.9, 145.4, 146.0, 146.9, 147.3, 149.8, 151.9, 157.4, 167.6, 171.9, 172.3. HR-MS (ESI-TOF): *m/z* calcd for $C_{64}H_{71}N_4O_{14}S_2$ ([M+H]+) 1183.4403, found 1183.4357.

Synthesis of SNC6DC: SN-38×C6 (50.0 mg, 0.102 mmol), triphosgene (12.0 mg, 0.040 mmol) and DMAP (61 mg, 0.499 mmol) were dissolved in CH_2Cl_2 (2.0 mL). The reaction mixture was stirred at 25 °C for 15 min, then 2,2'-dithiodiethanol (2-HD) (6.5 mg, 0.035 mmol) in CH_2Cl_2 (1.0 mL) was added sequentially. The reaction mixture was stirred overnight at 25 °C, followed by it was diluted with CH_2Cl_2, filtered and washed with saturated aqueous solution of NH_4Cl and brine simultaneously. The organic layer were separated, mixed and dried with $MgSO_4$. Concentration *in vacuo* afforded a residue, which was purified by silica gel column chromatography (Hexane/EtOAc = 3/1 → 0/100), then, further purified by silica gel column chromatography ($CHCl_3$/Acetone = 100/0 → 5/1) to get SNC6DC (22.0 mg, 0.0185 mmol, 46%) as a pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 0.96 (6H, t, J = 7.2 Hz), 1.38–1.44 (7H, m), 1.82 (2H, quin, J = 5.6 Hz), 2.05–2.11 (1H, m), 2.17–2.22 (1H, m), 2.66 (2H, t, J = 7.4 Hz), 2.78 (2H, t, J = 6.2 Hz), 3.15 (2H, q, J = 7.7 Hz), 3.88–4.00 (2H, m), 5.26–5.32 (2H, m), 5.35 (1H, d, J = 17.2 Hz), 5.80 (1H, d, J = 17.2 Hz), 7.21 (1H, s), 7.55 (1H, dd, J = 9.2, 2.4 Hz), 7.82 (1H, d, J = 2.4 Hz), 8.17 (1H, d, J = 9.2 Hz). ^{13}C NMR (100 MHz, $CDCl_3$, δ): 7.59, 13.9, 14.0, 22.3, 23.2, 24.5, 31.3, 31.8, 34.4, 36.9, 49.4, 66.0, 67.1, 77.9, 95.6, 114.7, 119.8, 125.6, 127.5, 127.6, 131.7, 145.4, 145.5, 147.1, 147.2, 149.7, 151.9, 153.2, 157.2, 167.3, 172.2. HR-MS (ESI-TOF): *m/z* calcd for $C_{62}H_{67}N_4O_{16}S_2$ ([M+H]+) 1187.3988, found 1187.3962.

Synthesis of SNC6DC (without S-S): SN-38×C6 (155.5 mg, 0.317 mmol), triphosgene (37.9 mg, 0.128 mmol) and DMAP (181.5 mg, 1.49 mmol) were dissolved in CH_2Cl_2 (6.1 mL). The reaction mixture was stirred at 25 °C for 15 min, then 1,6-hexanediol (18.7 mg, 0.158 mmol) in CH_2Cl_2 (1.0 mL) were added sequentially. The reaction mixture was stirred overnight at 25 °C, followed by it was diluted with CH_2Cl_2, filtered and washed with saturated aqueous solution of NH_4Cl and brine simultaneously. The organic layer were separated, mixed and dried with $MgSO_4$. Concentration in vacuo afforded a residue, which was purified by silica gel column chromatography ($CHCl_3$/Acetone = 100/0 → 5/1) to get SNC6DC (without S-S) (35.9 mg, 0.0312 mmol, 20%) as a pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 0.95–1.00 (6H, m), 1.31–1.61 (11H, m), 1.78–1.86 (2H, m), 2.09–2.15 (1H, m), 2.23–2.29 (1H, m), 2.66 (2H, t, *J* = 7.4 Hz), 3.15 (2H, q, *J* = 7.7 Hz), 3.86–3.96 (2H, m), 5.25 (2H, s), 5.38 (1H, d, *J* = 17.2 Hz), 5.73 (1H, d, *J* = 17.2 Hz), 7.25 (1H, s), 7.56 (1H, dd, *J* = 9.2, 2.4 Hz), 7.82 (1H, d, *J* = 2.4 Hz), 8.20 (1H, d, *J* = 9.2 Hz). ^{13}C NMR (125 MHz, $CDCl_3$, δ): 7.72, 14.0, 22.4, 23.3, 24.7, 25.1, 28.4, 31.4, 32.1, 34.5, 49.4, 67.2, 68.8, 77.7, 95.9, 114.7, 120.2, 125.7, 127.4, 127.6, 131.9, 145.5, 145.9, 147.1, 147.3, 149.9, 151.9, 153.8, 157.4, 167.6, 172.3. HR-MS (ESI-TOF): *m/z* calcd for $C_{64}H_{71}N_4O_{16}$ ([M+H]+) 1151.4860, found 1151.4818.

Synthesis of SNBocDC: Boc-SN-38 (1476.0 mg, 3.00 mmol), triphosgene (356.0 mg, 1.20 mmol), 2-HD (193.0 mg, 1.25 mmol) and DMAP (1830.0 mg, 15.0 mmol) were dissolved in CH_2Cl_2 (50.0 mL) at 0°C. The reaction mixture was stirred at 0 °C for 1 hour, 2-HD (193.0 mg, 1.25 mmol) and triphosgene (178.0 mg, 0.60 mmol) were added sequentially. The reaction mixture was stirred 2 hours, followed by it was diluted with $CHCl_3$, filtered and washed with saturated aqueous solution of NH_4Cl and brine simultaneously. The organic layer were separated, mixed and dried with $MgSO_4$. Concentration *in vacuo* afforded a residue, which was purified by recrystallization by MeOH. The obtained residue was washed MeOH to get SNBocDC (1269.0 mg, 1.28 mmol, 71%) as a pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 0.949 (3H, t, *J* = 7.4 Hz), 1.39 (3H, t, *J* = 7.8 Hz), 1.60 (9H, s), 2.03–2.23 (2H, m), 2.77 (2H, t, *J* = 6.2 Hz), 3.15 (q, *J* = 7.7 Hz), 3.85–4.00 (2H, m), 5.25 (2H, d, *J* = 5.6 Hz), 5.34 (1H, d, *J* = 17.2 Hz), 5.80 (1H, d, *J* = 17.2 Hz), 7.20 (1H, s), 7.65 (1H, dd, *J* = 9.2, 2.4 Hz), 7.89 (1H, d, *J* = 2.4 Hz), 8.16 (1H, d, *J* = 9.2 Hz). ^{13}C NMR (125 MHz, $CDCl_3$, δ): 7.69, 14.0, 23.3, 27.9, 32.0, 37.1, 49.5, 66.2, 67.2, 78.0, 84.5, 95.7, 114.4, 120.0, 125.4, 127.6, 127.7, 131.8, 145.6, 145.7, 147.27, 147.31, 150.1, 151.6, 152.1, 153.3, 157.3, 167.4. HR-MS (ESI-TOF): *m/z* calcd for $C_{60}H_{63}N_4O_{18}$ ([M+H]+) 1191.3573, found 1191.3545.

Synthesis of SNC0DC: SNBocDC (723.0 mg, 0.607 mmol) was dissolved in TFA solution (22mL, 10% TFA in CH_2Cl_2). The reaction mixture was stirred for 3.5 hours at 25 °C. Completion of reaction was monitored with TLC. The reaction mixture was concentrated under vacuum to get yellow viscous liquid. A portion of MeOH was added to get yellow precipitate.

The precipitate was sonicated, filtered, and wash with MeOH then $CHCl_3$ many times, which was dried under vacuum to get pure SNC0DC (596.0 mg, 0.601 mmol, 99%) as a pale yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 0.881 (3H, t, *J* =7.4 Hz), 1.26 (3H, t, *J* = 7.6 Hz), 2.08–2.15 (2H, m), 2.90–2.99 (2H, m), 3.04 (2H, q, *J* = 7.5 Hz), 5.23 (2H, s). 5.5 (2H, d, *J* = 3.2 Hz), 6.91 (1H, s). 7.36–7.38 (2H, m), 7.96 (1H, d, *J* = 10 Hz), 10.3 (1H, br). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 7.53, 13.3, 22.3, 30.3, 36.2, 49.4, 66.1, 66.5, 79.2, 93.4, 104.7, 118.1, 122.4, 127.8, 128.2, 131.5, 142.7, 143.5, 144.8, 147.2, 148.5, 152.7, 156.5, 156.8, 167.1. HR-MS (ESI-TOF): *m/z* calcd for $C_{50}H_{47}N_4O_{14}S_2$ ([M+H]+) 991.2525, found 991.2494.

Synthesis of SNC2DC: SNC0DC (200.0 mg, 0.202 mmol), TEA (101.0 mg, 0.998 mmol), and DMAP (5.0 mg, 0.0409 mmol) were dissolved in CH_2Cl_2 (2.8 mL) at 0°C. Acetyl chloride (36 μL, 0.507 mmol) were added at 0°C. The reaction mixture was stirred overnight, followed by it was diluted with $CHCl_3$, filtered and washed with water and brine simultaneously. The organic layer were separated, mixed and dried with $MgSO_4$. Concentration in vacuo afforded a residue, which was purified by silica gel column chromatography ($CHCl_3$/Acetone = 100/0 → 9/1) to get SNC2DC (80.8 mg, 0.075 mmol, 37%) as a pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 0.953 (3H, t, *J* = 7.4 Hz), 1.37 (3H, t, *J* = 7.6 Hz), 2.03–2.25 (2H, m), 2.40 (3H, s), 2.78 (2H, t, *J* = 6.2 Hz), 3.14 (2H, q, *J* = 7.7 Hz), 3.87–4.02 (2H, m), 5.25 (2H, d, *J* = 5.2 Hz), 5.34 (1H, d, *J* = 17.2 Hz), 5.78 (1H, d, *J* = 16.8 Hz), 7.20 (1H, s), 7.55 (1H, dd, *J* = 9.2, 2.8 Hz), 7.82 (1H, d, *J* = 2.4 Hz), 8.16 (1H, d, *J* = 9.2 Hz). ^{13}C NMR (125 MHz, $CDCl_3$, δ): 7.68, 14.0, 21.3, 23.3, 32.0, 37.0, 49.5, 66.2, 67.2, 78.0, 95.7, 114.9, 120.0, 125.6, 127.6, 127.7, 131.9, 145.5, 145.7, 147.28, 147.34, 149.7, 152.1, 153.3, 157.3, 167.4, 169.4. HR-MS (ESI-TOF): *m/z* calcd for $C_{54}H_{51}N_4O_{16}S_2$ ([M+H]+) 1075.2736, found 1075.2726.

General method for synthesis of SNCnDC (n = 4, 6, 8, 10): SNC0DC (200.0 mg, 0.202 mmol) was dissolved in CH_2Cl_2 (2.5 mL, 0.08 M), and then fatty acid (0.501 mmol), EDC·HCl (84.3 mg, 0.440 mmol) and DMAP (4.89 mg, 0.040 mmol) at room temperature. The reaction mixture was stirred for 4 hours at 25 °C, followed by it was diluted with CH_2Cl_2, filtered and washed with saturated aqueous solution of NH_4Cl and brine simultaneously. The organic layer were separated, mixed and dried with $MgSO_4$. Concentration *in vacuo* afforded a residue, which was purified by silica gel column chromatography ($CHCl_3$/Acetone = 100/0 → 5/1) to get SNCnDC.

SNC4DC: SNC0DC (500.0 mg, 0.504 mmol), Butyric acid (110.0 mg, 1.25 mmol), EDC·HCl (194.0 mg, 1.012 mmol) and DMAP (13.0 mg, 0.106 mmol) afford SNC4DC (350.0 mg, 0.309 mmol, 62%) as a pale-yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 0.958 (3H, t, *J* = 7.4 Hz), 1.10 (3H, t, *J* = 7.4 Hz), 1.40 (3H, t, *J* = 7.4 Hz), 1.81–1.90 (2H, m), 2.05–2.12 (1H, m), 2.17–2.24 (1H, m), 2.65 (2H, t, *J* = 7.4 Hz), 2.78 (2H, t, *J* = 6.2 Hz), 3.15 (2H, q, *J* = 7.6 Hz), 3.87–3.93 (1H, m), 3.96–4.02 (1H, m), 5.27 (2H, d, *J* = 4.8 Hz), 5.30 (1H, d, *J* = 17.2 Hz), 5.80 (1H,

d, J = 17.2 Hz), 7.21 (1H, s), 7.55 (1H, dd, J = 9.2, 2.4 Hz), 7.56 (1H, d, J = 2.4 Hz), 8.17 (1H, d, J = 9.2 Hz). ^{13}C NMR (100 MHz, $CDCl_3$, δ): 7.58, 13.7, 13.9, 18.4, 23.1, 31.8, 36.2, 36.9, 49.4, 66.0, 67.1, 77.9, 95.5, 114.7, 119.8, 125.6, 127.48, 127.54, 131.7, 145.4, 145.5, 147.1, 147.2, 149.7, 151.9, 153.2, 157.2, 167.3, 172.0. HR-MS (ESI-TOF): *m/z* calcd for $C_{58}H_{59}N_4O_{16}S_2$ ([M+H]+) 1131.3362, found 1131.3327.

SNC6DC: SNC0DC (164.2 mg, 0.166 mmol), Hexanoic acid (46.5 mg, 0.400 mmol), EDC·HCl (85.2 mg, 0.444 mmol) and DMAP (13.6 mg, 0.111 mmol) afford SNC6DC (155.6 mg, 0.131 mmol, 79%) as a pale-yellow solid.

SNC8DC: SNC0DC (100.0 mg, 0.101 mmol), Octanoic acid (58.0 mg, 0.402 mmol), EDC·HCl (42.0 mg, 0.219 mmol) and DMAP (2.5 mg, 0.020 mmol) afford SNC8DC (62.0 mg, 0.050 mmol, 50%) as a pale-yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 0.905 (3H, t, J = 6.8 Hz), 0.957 (3H, t, J = 7.4 Hz), 1.31–1.42 (11H, m), 1.77–1.83 (2H, m), 2.05–2.24 (2H, m), 2.66 (2H, t, J = 7.6 Hz), 2.78 (2H, t, J = 6.2 Hz), 3.15 (2H, q, J = 7.7 Hz), 3.88–4.00 (2H, m), 5.26 (2H, d, J = 5.2 Hz), 5.35 (1H, d, J = 17.2 Hz), 5.80 (1H, d, J = 17.2 Hz), 7.21 (1H, s), 7.55 (1H, dd, J = 9.2, 2.4 Hz), 7.82 (1H, d, J = 2.8 Hz), 8.17 (1H, d, J = 9.2 Hz). ^{13}C NMR (125 MHz, $CDCl_3$, δ): 7.65, 14.0, 14.2, 22.7, 23.2, 25.0, 29.0, 29.2, 31.7, 32.0, 34.5, 37.1, 49.5, 66.1, 67.2, 78.0, 95.6, 114.8, 120.0, 125.6, 127.60, 127.64, 131.8, 145.5, 145.6, 147.26, 147.28, 149.8, 152.0, 153.3, 157.3, 167.4, 172.2. HR-MS (ESI-TOF): *m/z* calcd for $C_{66}H_{75}N_4O_{16}S_2$ ([M+H]+) 1243.4614, found 1243.4594.

SNC10DC: SNC0DC (103.2 mg, 0.104 mmol), Decanoic acid (52.1 mg, 0.302 mmol), EDC·HCl (55.4 mg, 0.289 mmol) and DMAP (6.5 mg, 0.053 mmol) afford SNC10DC (79.9 mg, 0.061 mmol, 59%) as a pale-yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 0.873 (3H, t, J = 7.0 Hz), 0.944 (3H, t, J = 7.6 Hz), 1.27–1.5 (15H, m), 1.76–1.82 (2H, m), 2.03–2.09 (1H, m), 2.17–2.23 (1H, m), 2.65 (2H, t, J = 7.6 Hz), 2.76 (2H, t, J = 6.2 Hz), 3.14 (2H, q, J = 7.7 Hz), 3.85–3.90 (1H, m), 3.93–3.98 (1H, m), 5.24 (2H, d, J = 5.2 Hz), 5.33 (1H, d, J = 17.2 Hz), 5.79 (1H, d, J = 17.2 Hz), 7.19 (1H, s), 7.53 (1H, dd, J = 9.2, 2.4 Hz), 7.80 (1H, d, J = 2.4 Hz), 8.15 (1H, d, J = 9.2 Hz). ^{13}C NMR (100 MHz, $CDCl_3$, δ): 7.69, 14.0, 14.2, 22.8, 23.3, 25.0, 29.3, 29.4, 29.6, 32.0, 34.6, 37.1, 49.5, 66.1, 67.2, 78.0, 95.7, 114.8, 120.0, 125.7, 127.6, 127.7, 131.8, 145.5, 145.7, 147.30, 147.32, 149.8, 152.1, 153.3, 157.3, 167.4, 172.3. HR-MS (ESI-TOF): *m/z* calcd for $C_{70}H_{83}N_4O_{16}S_2$ ([M+H]+) 1299.5240, found 1299.5193.

3. Experimental Procedure

Fabrication and characterization of the NPDs: The NPDs were fabricated using the reprecipitation method. The target compound (1 μmol of as the SN-38 monomer) in a good solvent (100 μL) was rapidly injected into stirred deionized water (10 mL) using a microsyringe at room temperature. The colloidal solution was deposited on a polycarbonate membrane (Whatman® Nucleopore Track-Etched Membranes) by filtration and characterized using FE-SEM. The size distribution and zeta potential were recorded at 25 °C.

In vitro cell-viability experiments: 100 μL of cells were seeded in 96-well plates at a density of 2×10^4 cells $well^{-1}$. After 24 hours, the supernatant was removed and 100 μL of different NPDs dispersion solutions (0.04–10 μм based on the SN-38 concentration) were added to the wells. The 96-well plate was kept in a CO_2 incubator for 48 hours, and the cell viability was determined using a WST-8 assay. The cell viability values were normalized to OD_{450}–OD_{620} for the untreated cells. Assays were performed in triplicate.

Drug release triggered by GSH: Aqueous dispersions of SNC4DC, SNC0DC, and SN-38×C4 equivalent to 0.1 mM of SN-38 were prepared. Aqueous dispersions of each SN-38 prodrug (500 μL) were added to 10 mM of GSH in a mixture of phosphate-buffered saline and ethanol (v/v = 70/30, pH = 7.4, 500 μL). The mixture was incubated at 37 °C in a water bath. After 1, 6, 24, and 48 hours, the reaction mixture (500 μL) was added to MeCN containing 3% acetic acid (500 μL), and the drug-release rate of the samples was evaluated using high-performance liquid chromatography (HPLC). The HPLC conditions for all samples are described here; column: reverse-phase column (Unison UK-C18, 150 × 4.6 mm); column temperature: 40 °C; mobile phase: gradient elution using a mixture of 0.1% formic acid in water and 0.1% formic acid in MeCN with volume ratio (v/v) from 40/60 maintained for 5 min, then linearly increased to 10/90 for 2 min, and maintained for another 8 min; flow rate: 1 mL min^{-1}; injection volume: 10 μL.

Drug release triggered by esterase: To 200 μL of phosphate-buffered saline (pH = 7.4), porcine-liver-esterase powder (10 units) and a 0.4 mM aqueous dispersion of SNC4DC (800 μL) were added. The mixture was incubated at 37 °C in a water bath, and 60 μL of the sample was withdrawn at different time points (0.5, 1, 2, 4, and 6 hours). The samples were extracted with 540 μL of $CHCl_3$. The supernatant was diluted with MeCN, and the hydrolysis rate of the samples was evaluated using HPLC.

Fabrication of 6.3 mM NPDs dispersions with surfactant: The NPDs were fabricated using the reprecipitation method. Water was purified to 18.2 MΩ•cm using a PURELAB flex 3. 750 μL of SNC4DC in THF (46.7 mM) was rapidly injected into stirred deionized water (4.25 mL)

using a microsyringe at room temperature. The THF solution also contained the same weight of Polysorbate 80 (PS80) surfactant as SNC4DC. THF was removed from the fabricated NPDs dispersion under reduced pressure followed by volume adjustment to 5 mL with deionized water (7 mM). The fabricated NPDs dispersion was mixed with a 9% NaCl aqueous solution at a 9:1 ratio to achieve a 6.3 mM NPDs dispersion.

Animal experiments: BALB/c nu/nu mice (5-week-old, female) were obtained from Charles River Laboratories Japan, Inc. Nude mice were kept in a natural light/dark cycle under constant temperature and humidity conditions (22 ± 4°C, 50 ± 10%). The Ethical Committee of the Graduate School of Medicine, Tohoku University, approved the protocol under No. 2013MdA-377. The methods were carried out in accordance with the approved guidelines.

Antitumor activity: An *in vivo* experiment was performed using HCT-116 xenograft mice. 5.0×10^6 cells of HCT-116 cells suspended in PBS (100 μL) were injected in the both flanks under anesthesia. All tumors were grown at the subcutaneous site. Tumor-bearing mice were randomized into groups of five prior to initiating therapy. Mice bearing bilateral subcutaneous tumors each received the SNC4DC NPDs when the tumors were approximately 0.3–1.0 cm in diameter. In the control groups, the animals received an injection of saline or irinotecan. All mice were injected intravenously with 100 μL of each sample. The amount of injected drug was calculated by assuming that each mouse weight 25 g, and the drug based on SN-38 was injected 10mg/kg of mouse weight. For the first 20 days after starting the drug treatment, the two perpendicular diameters of the tumor were determined in 2-day intervals using vernier calipers. The volume of the tumor (V) was calculated according to the following equation: $V = 0.5 \times L \times W^2$. L (mm) is the longest diameter and W (mm) is the diameter perpendicular to L.
Statistical analyses were performed using GraphPad Prism version 9.2. The statistical significance of *in vivo* experiments was evaluated using a one-way analysis of variance (ANOVA) test with Tukey's multiple comparisons test. The threshold for significance was $p < 0.05$.

Red blood cell (RBC) count: 100 μL of each anticancer drug was intravenously administered to the mice every other day for 10 days. 70 μL of blood was collected the day after the last drug administration, and the collected blood was diluted 200 times with Hayem's solution. The number of RBCs was determined by microscopic count in a Bürker-Türk counting chamber.

White blood cell (WBC) count: 100 μL of each anticancer drug was intravenously administered to the mice every other day for 10 days. 70 μL of blood was collected the day after the last drug administration, and the collected blood was diluted 10 times with Turk's solution. The number

of WBCs was determined by microscopic count in a Bürker-Türk counting chamber.

4. Supplementary Figures

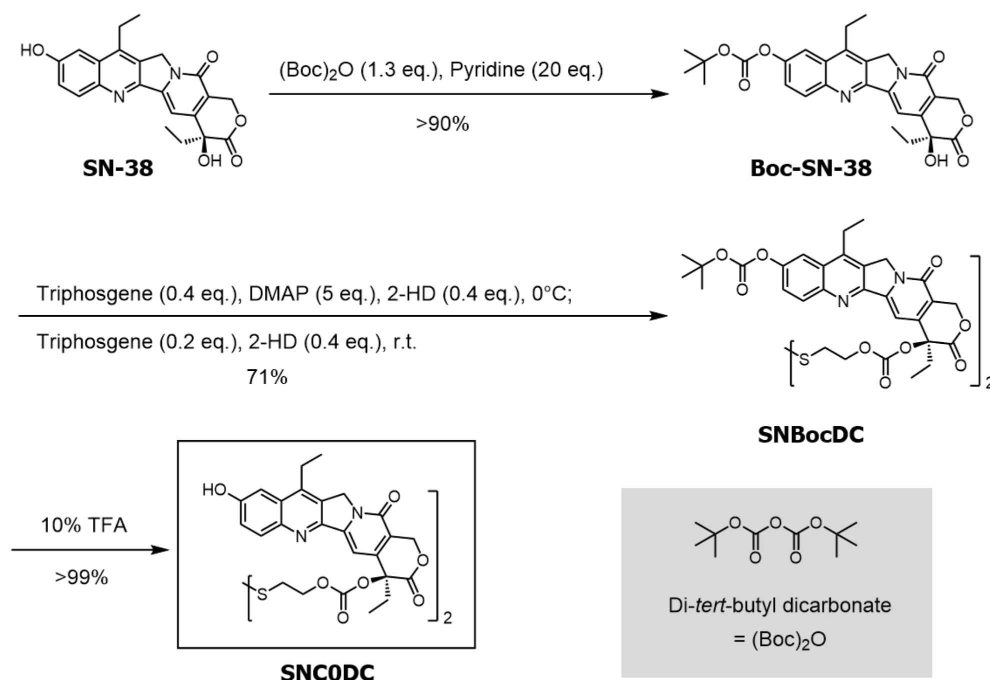


Figure S1. Synthesis route of SNC0DC.

Table S1. Esterification of SNC0DC to SNCnDC (n = 2, 4, 6, 8, 10).

Entry	Substituent (n)	Condition	Yield [%][a)]
1	2	Acetyl chloride (2.5 eq.), TEA (5.0 eq.), DMAP (0.2 eq.)	37
2	4	Butyric acid (2.5 eq.), EDC (2.5 eq.), DMAP (0.2 eq.)	62
3	6	Hexanoic acid (2.5 eq.), EDC (2.2 eq.), DMAP (0.2 eq.)	79
4	8	Octanoic acid (4.0 eq.), EDC (2.2 eq.), DMAP (0.2 eq.)	50
5	10	Decanoic acid (2.5 eq.), EDC (2.2 eq.), DMAP (0.2 eq.)	59

a) Isolated yield

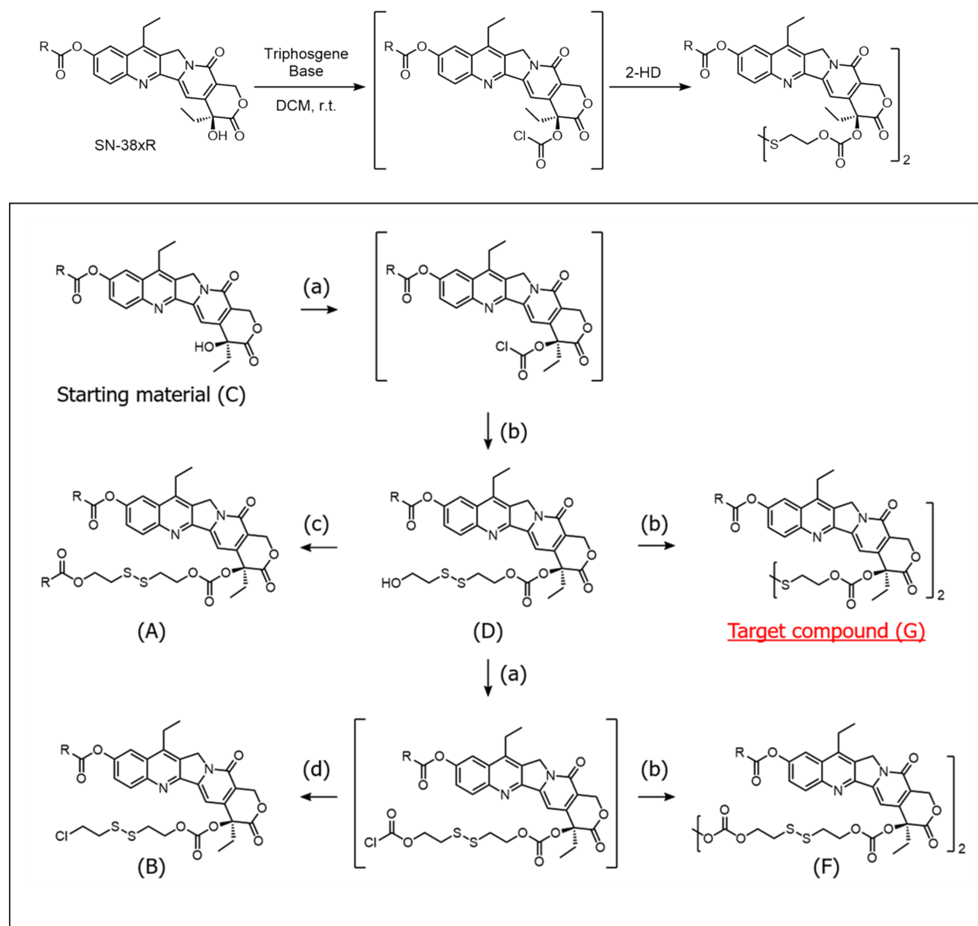


Figure S2. Short-step synthetic route and the obtained byproducts. Reaction mechanism. (a) Formation of chloroformate; (b) Formation of carbonate; (c) Transesterification; (d) Introduction of chlorine.

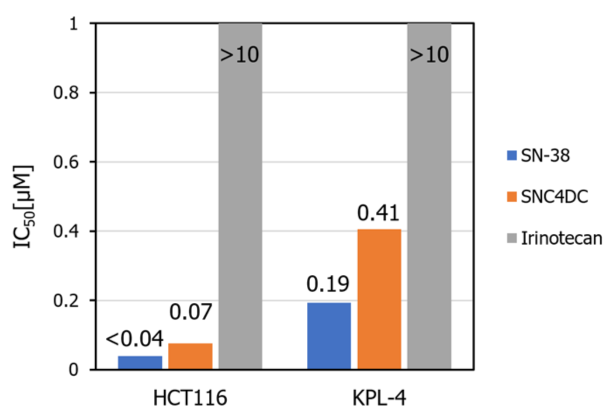


Figure S3. Difference in cytotoxicity of SN-38, SNC4DC NPDs and irinotecan. The cell viability of SNCnDC were evaluated in HCT-116 and KPL-4 cell lines. Cell viability was evaluated by WST-8 assay at 48 hours after incubation with varying concentration (converted as SN-38).

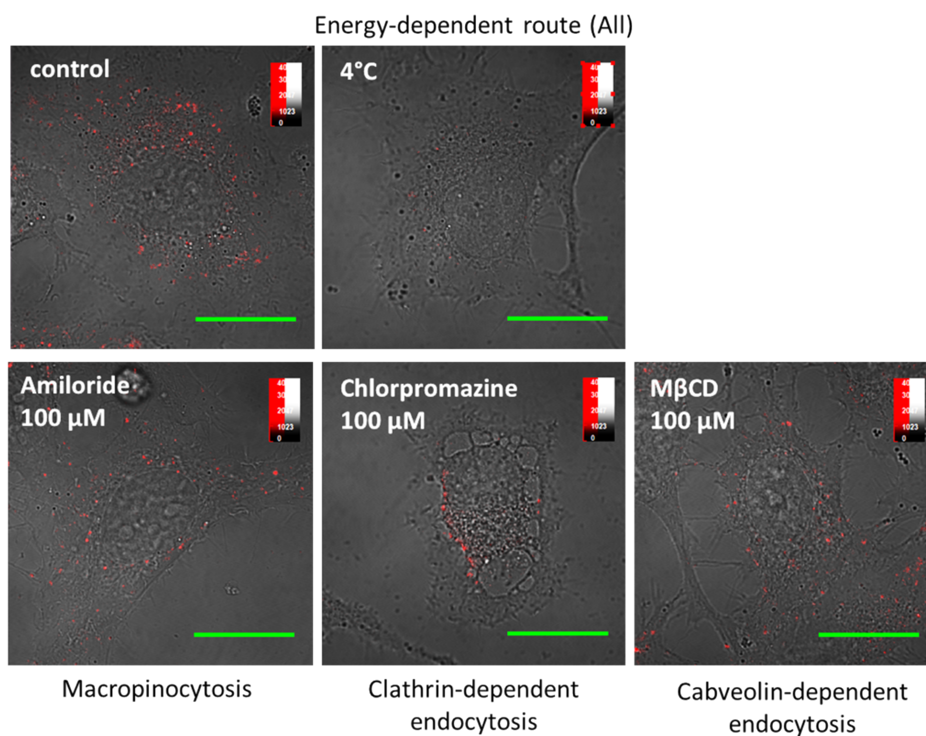


Figure S4. Intracellular dynamics of SNC4DC NPDs. The possible cellular uptake pathways of SNC4DC NPDs were investigated by observing HeLa cancer cells pretreated with different endocytosis inhibitor using confocal laser scanning microscopy (CLSM) spectroscopy. HeLa cells were pre-incubated 30 min with inhibitor in HBSS solution. SNC4DC NPDs (10 μм) were added to cells and further incubate for 2 h. SNC4DC NPDs is shown in red. Scale bar 20 μm.

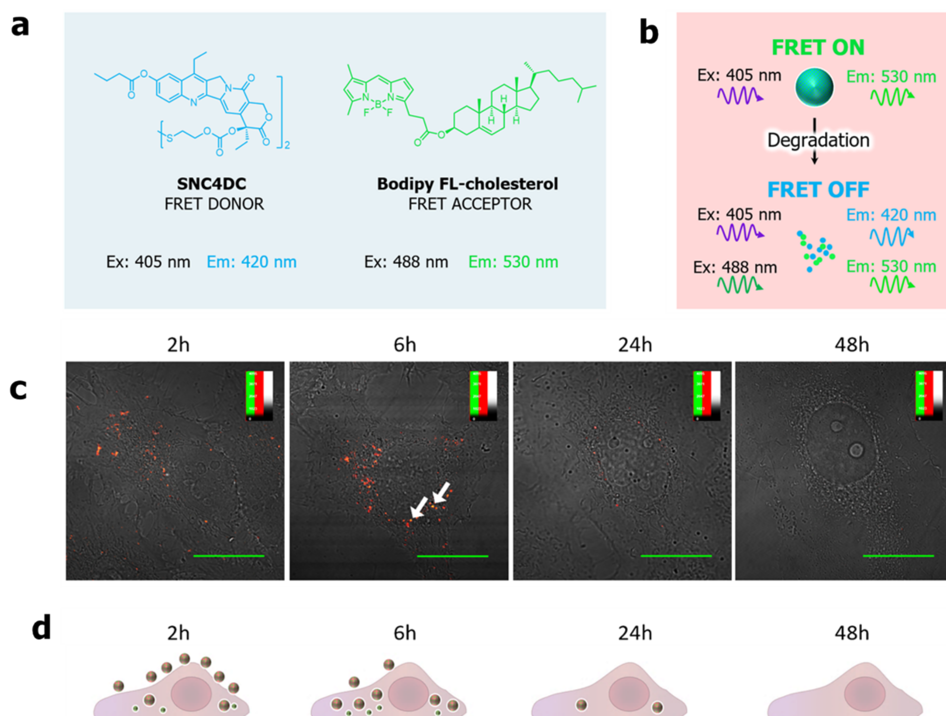


Figure S5. Investigation of cellular uptake pathways. (a) Chemical structure of coprecipitated compounds, and their excitation and emission wavelengths. (b) Differences in emission wavelengths between the nanoparticle and molecular states. In the NPDs state, BPFL-cholesterol is in close proximity with SNC4DC, enabling FRET to occur. In contrast, the dissolution of SNC4DC or BPFL-cholesterol molecules increases the distance between FRET donor and acceptor, quenching the FRET signal. (c) Internalization and intracellular degradation of FRET-SNC4DC NPDs. CLSM images of HeLa cells with FRET-SNC4DC NPDs: measurement taken at 2, 6, 24 and 48 hours. SNC4DC is shown in green, BPFL-cholesterol is shown in red. Scale bars: 10 µm. (d) Schematic representation of FRET-SNC4DC NPDs intracellular dynamics. It is clear from the local FRET signal of 530 nm emission that SNC4DC NPD was taken up into the cell with nanoparticle state within 6h. Then, the dissolution of compounds from particles state occurred spontaneously, due to both signals of FRET-SNC4DC NPDs and SNC4DC monomer (yellow spots indicated with arrows) can be observed. At 24 hours, the decrease of signals derived from both FRET-SNC4DC NPDs and SNC4DC monomer was observed. This observation could be related to the hydrolysis of the ester bond at the phenol hydroxy group which quenches the fluorescence of SNC4DC. At 48 hours, the morphology of HeLa cells changed and only a small number of cells were left attached on the dishes. Signals of NPDs can no longer be detected due to the metabolism of prodrug.

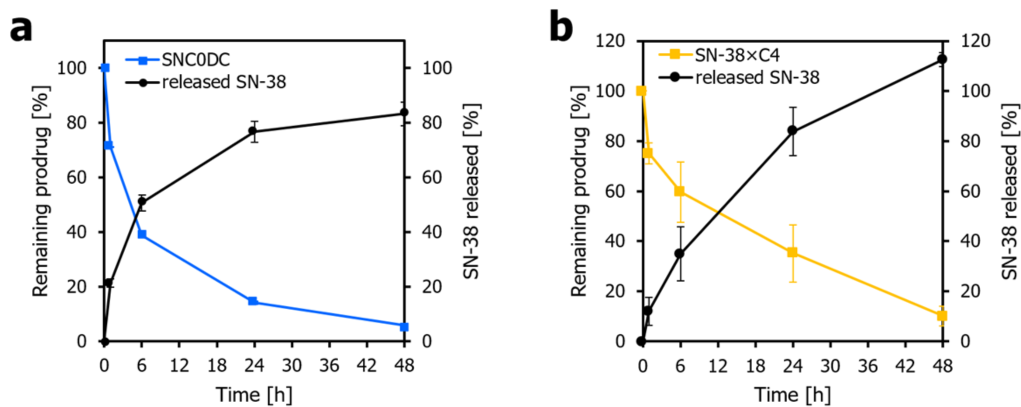


Figure S6. Drug release behavior under 5 mM GSH conditions. (a) SNC0DC and (b) SN-38×C4. The data are shown as the mean ± standard deviation (n = 3 independent experiments).

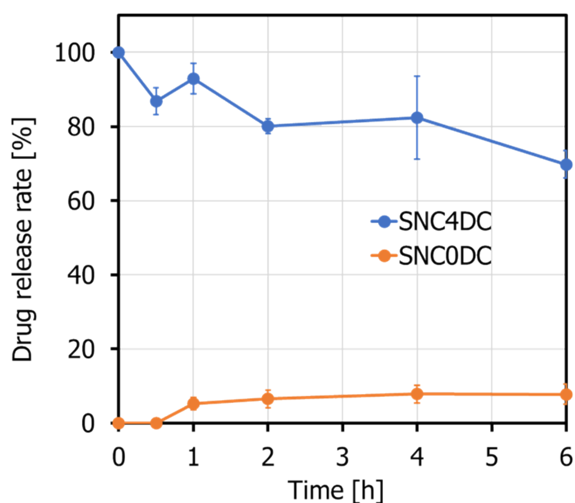
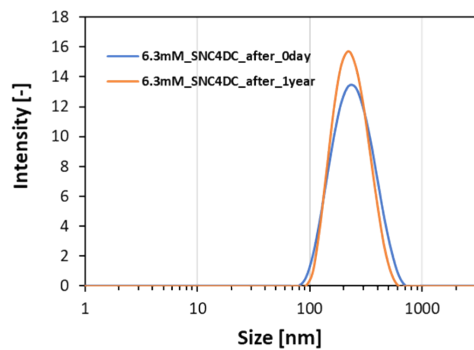
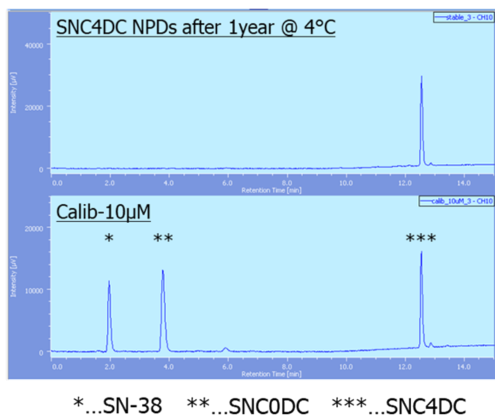


Figure S7. Determination of the hydrolysis susceptibility of SNC4DC in the presence of the porcine liver esterase (PLE). This experiment was measured at a PLE concentration of 10U. The data are shown as mean ± standard deviation (n = 3 independent experiments).





*...SN-38 **...SNC0DC ***...SNC4DC

Figure S8. Evaluation of long-term stability of high concentrated SNC4DC NPDs. (a) Size dispersion profile measured immediately after production and one year later. (b) HPLC data after 1 year of storage.

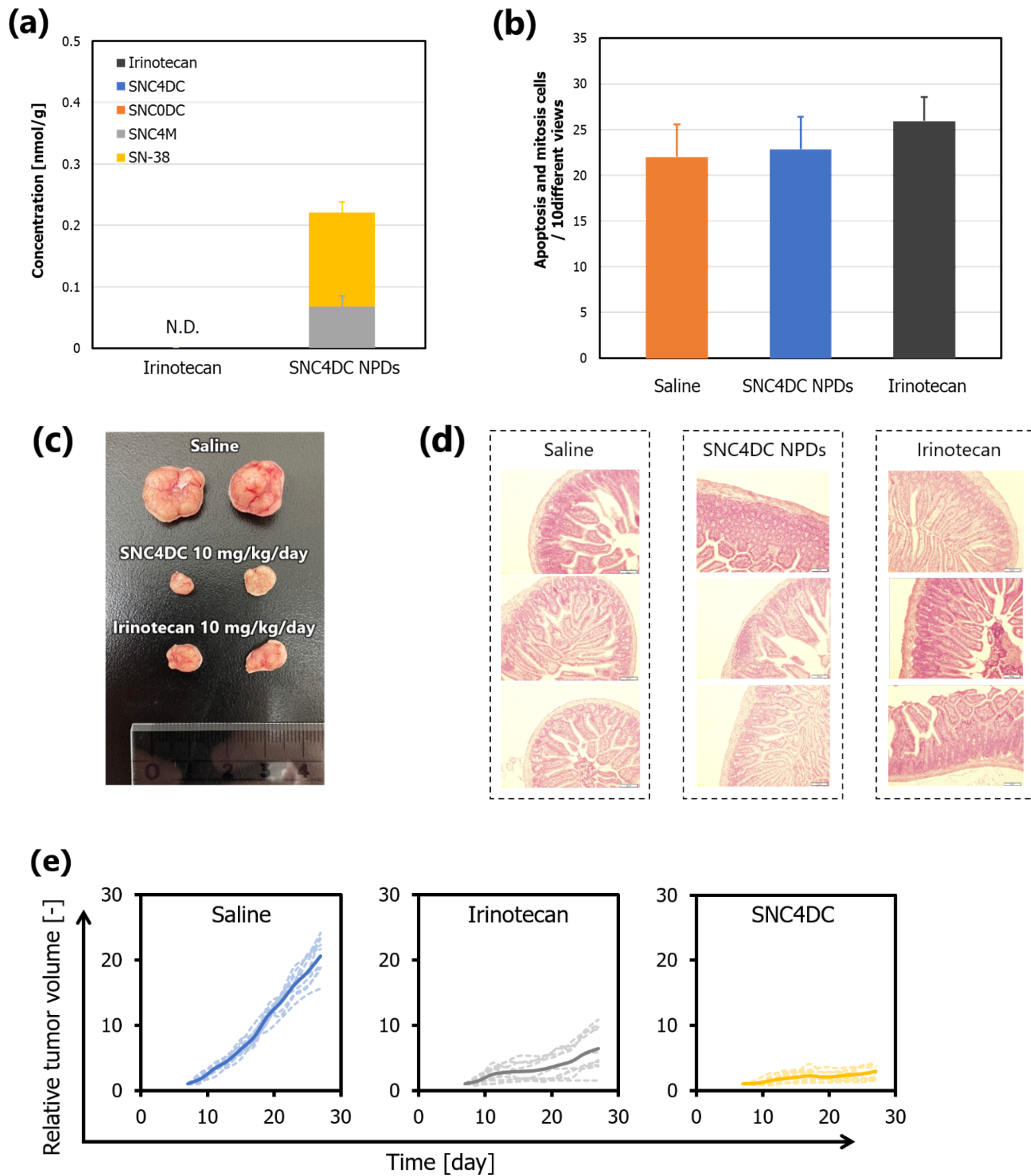
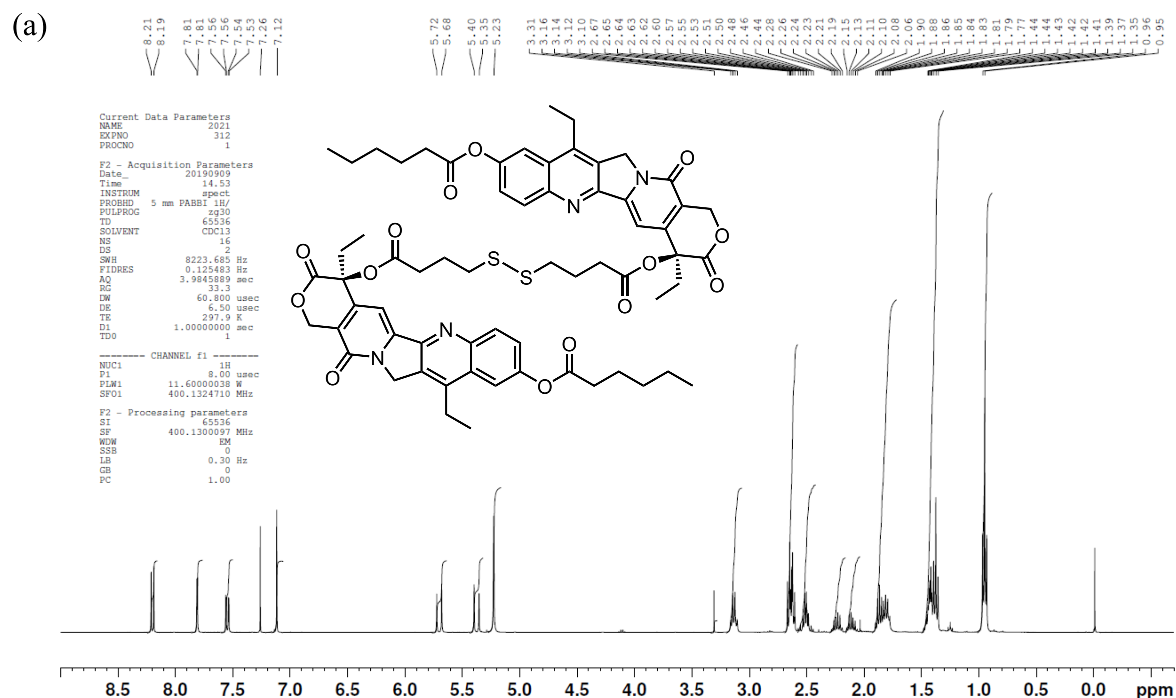


Figure S9 (a) Amount of SN-38 derivatives present in tumor after 48 hours. (b) Evaluation of side effect on small intestine. Hematoxylin-eosin staining of small intestinal tissue from mice at 14 days after five intravenous injections of anticancer drugs. The data are shown as mean ± standard deviation (n = 3 independent experiments). (c) Tumor image with the real scale bar. (d) HE-staining images. Objective (10X), eyepiece (15X). (e) Individual tumor growth curve (dashed line) and their average (Solid line).

5. NMR spectrum

(a)



(b)

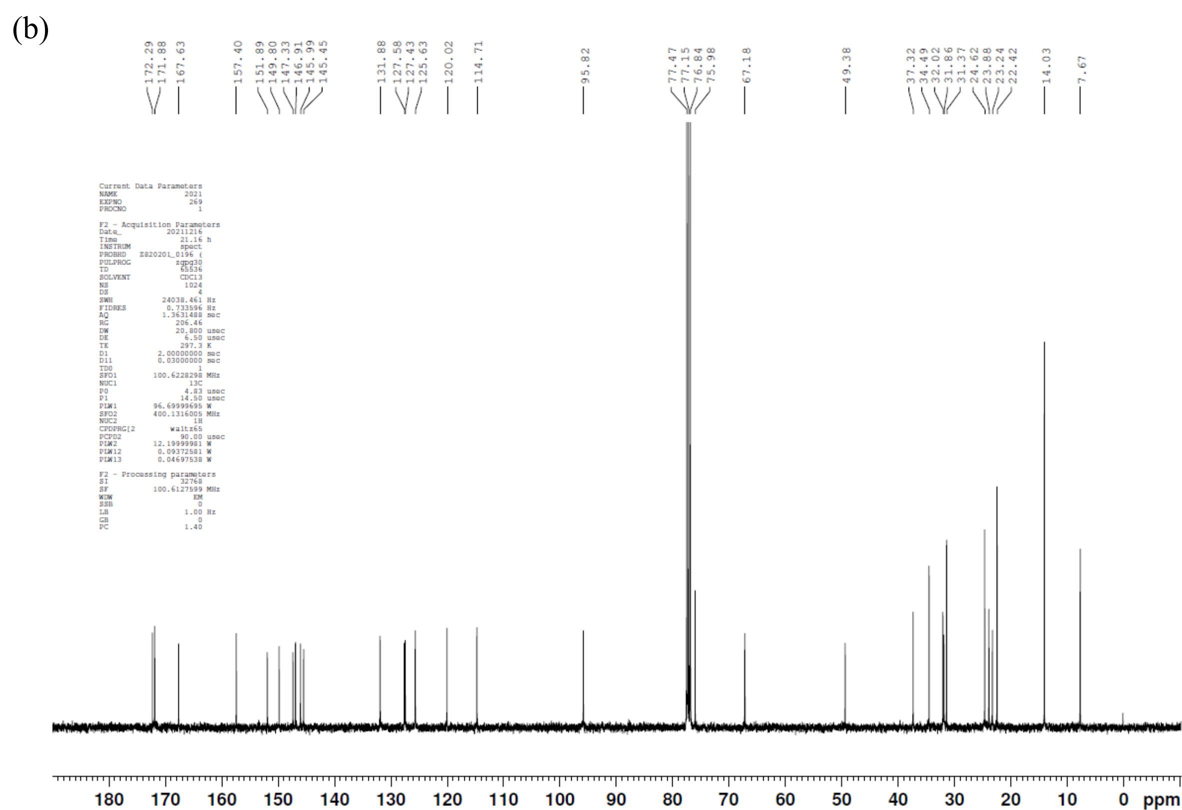
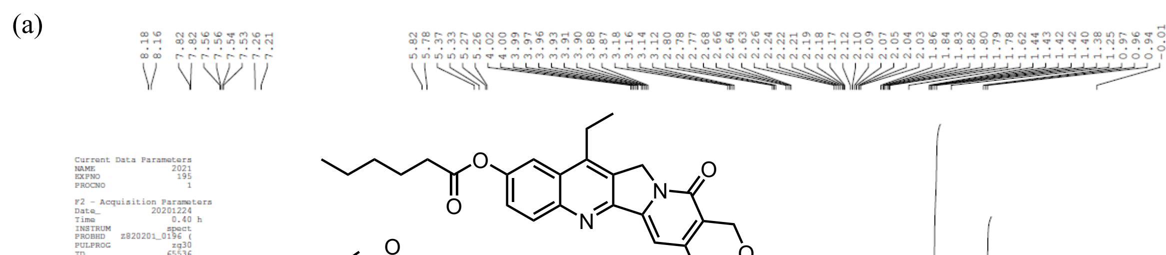


Figure S10. NMR spectrum of SNC6DE.

(a) ^{1}H NMR ($CDCl_3$, 400 MHz), (b) ^{13}C NMR ($CDCl_3$, 100 MHz).

(a)



(b)

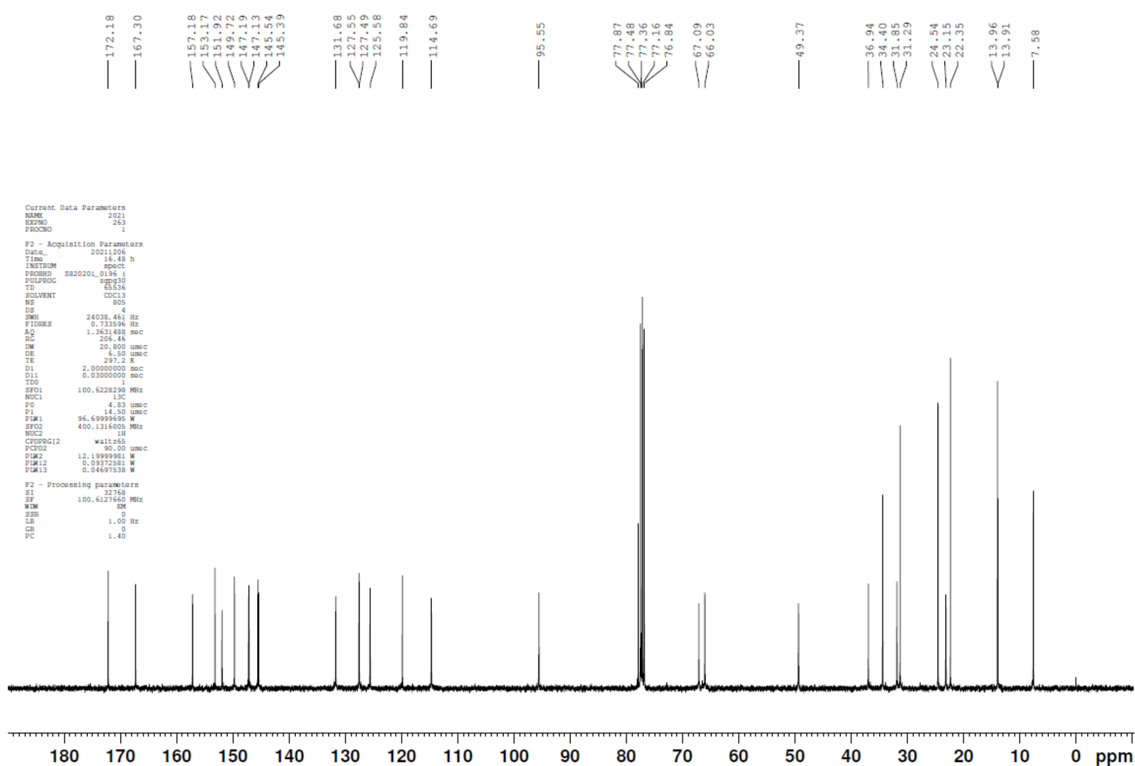
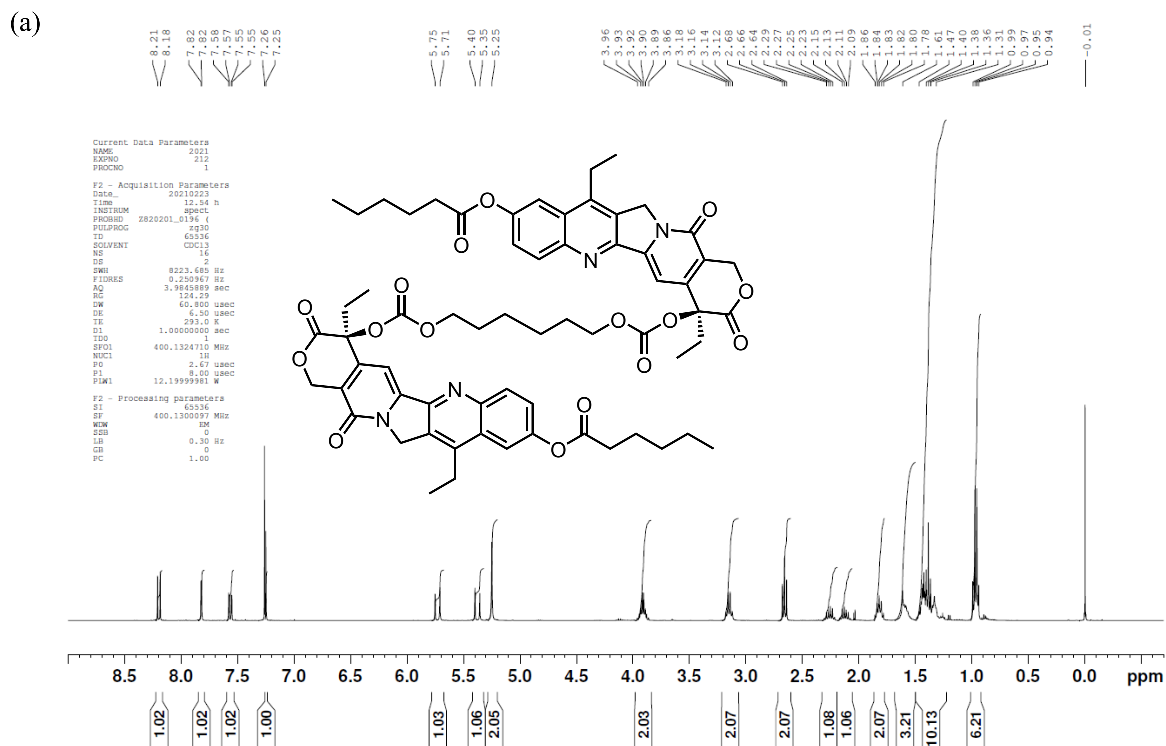


Figure S11. NMR spectrum of SNC6DC.

(a) ^{1}H NMR ($CDCl_3$, 400 MHz), (b) ^{13}C NMR ($CDCl_3$, 100 MHz).

(a)



(b)

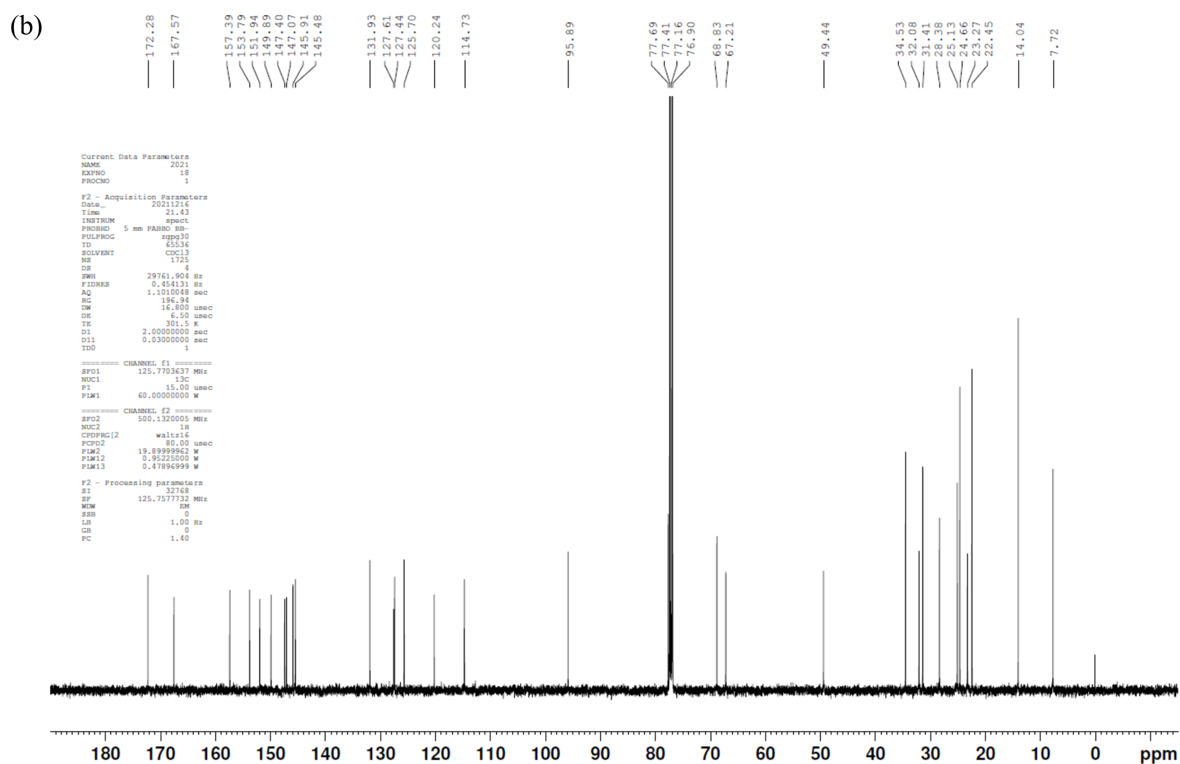
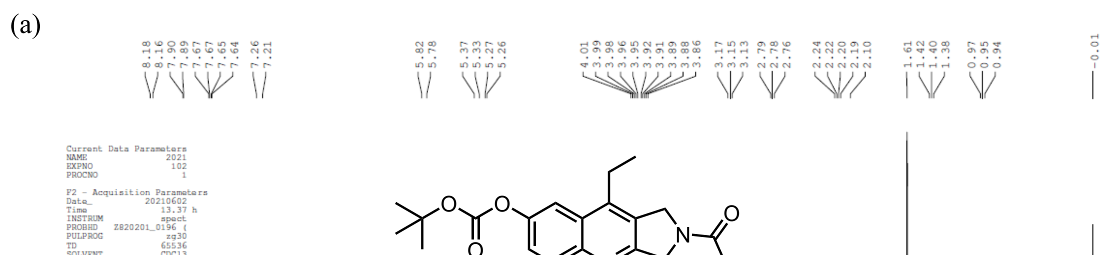


Figure S12. NMR spectrum of SNC6DC (without S-S).
(a) ^{1}H NMR ($CDCl_3$, 400 MHz), (b) ^{13}C NMR ($CDCl_3$, 125 MHz).

(a)



(b)

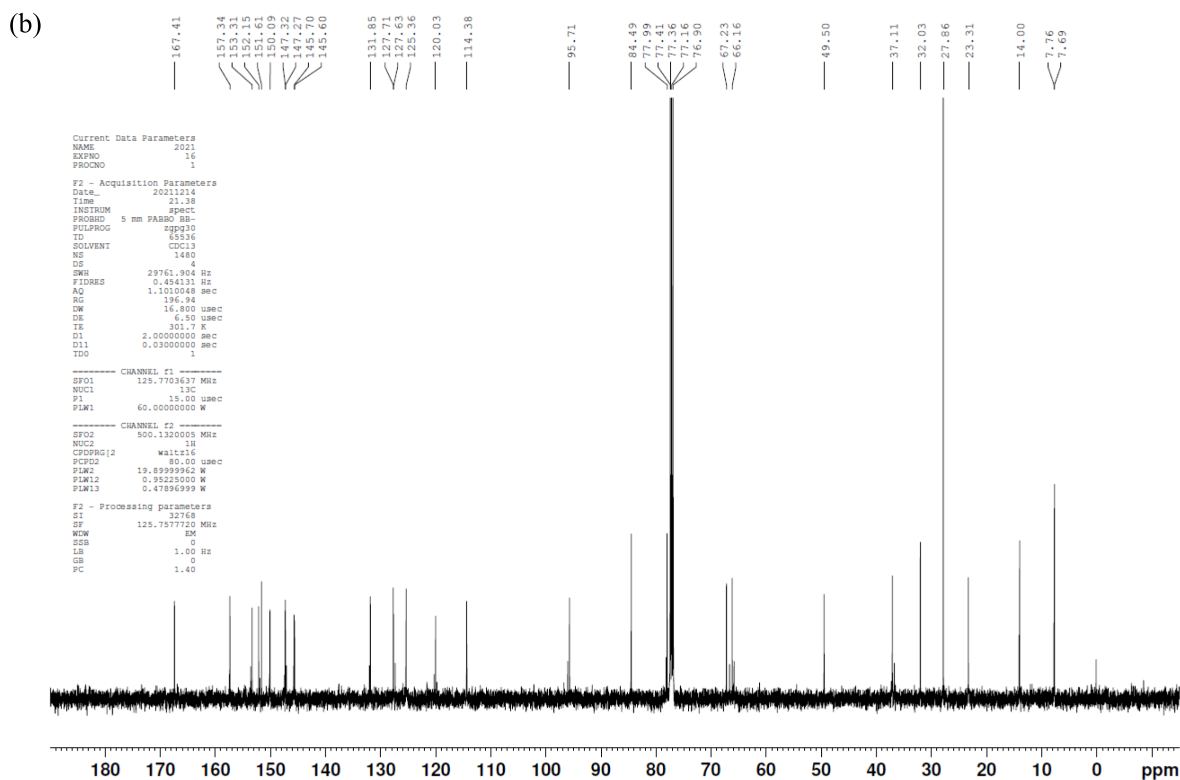
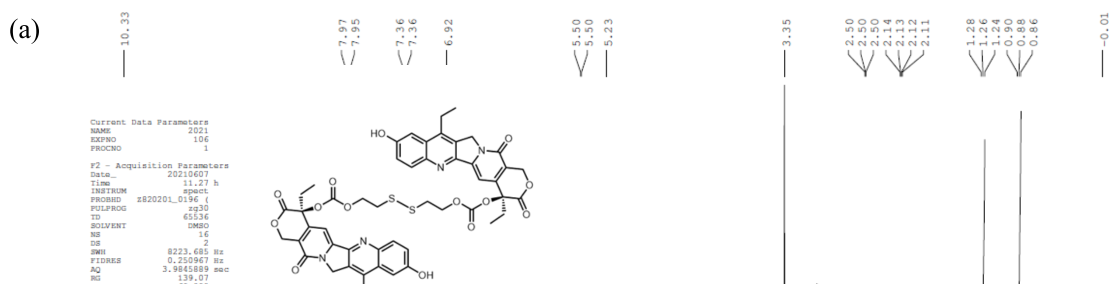


Figure S13. NMR spectrum of SNBocDC.

(a) ^{1}H NMR ($CDCl_3$, 400 MHz), (b) ^{13}C NMR ($CDCl_3$, 125 MHz).

(a)

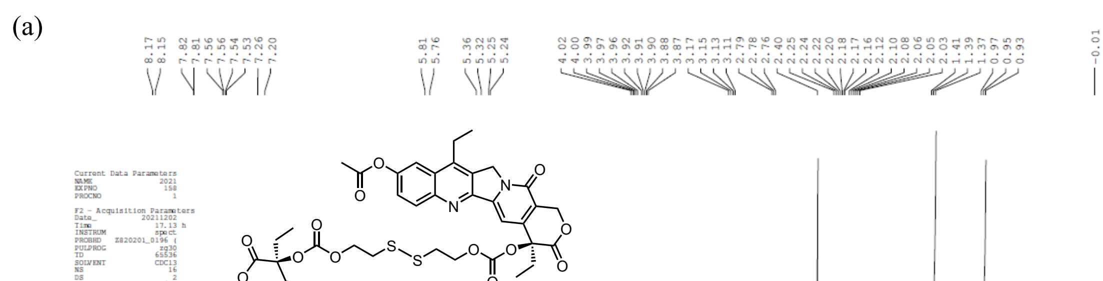


(b)

Figure S14. NMR spectrum of SNC0DC.

(a) ^{1}H NMR (DMSO, 400 MHz), (b) ^{13}C NMR (DMSO, 100 MHz).

(a)



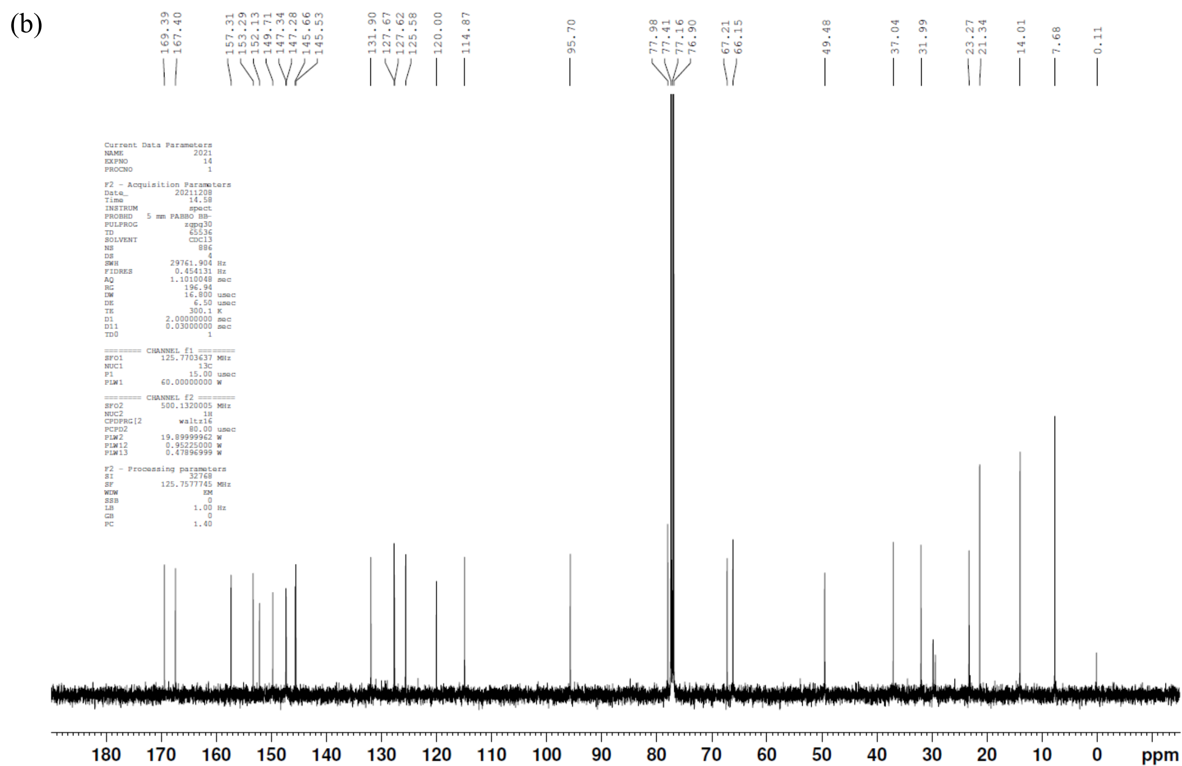
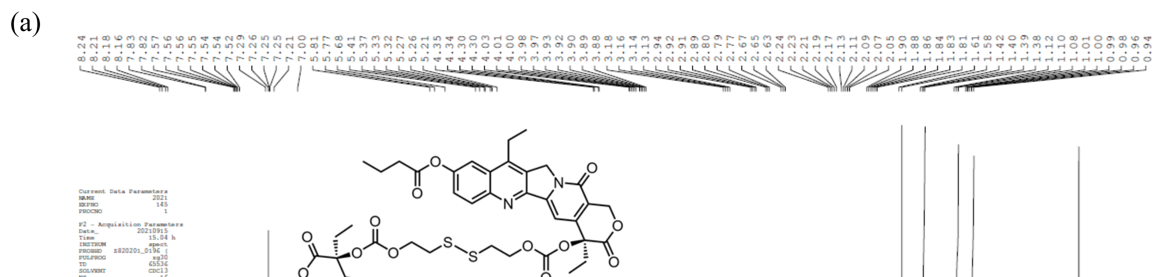


Figure S15. NMR spectrum of SNC2DC.

(a) ^{1}H NMR ($CDCl_3$, 400 MHz), (b) ^{13}C NMR ($CDCl_3$, 125 MHz).



(b)

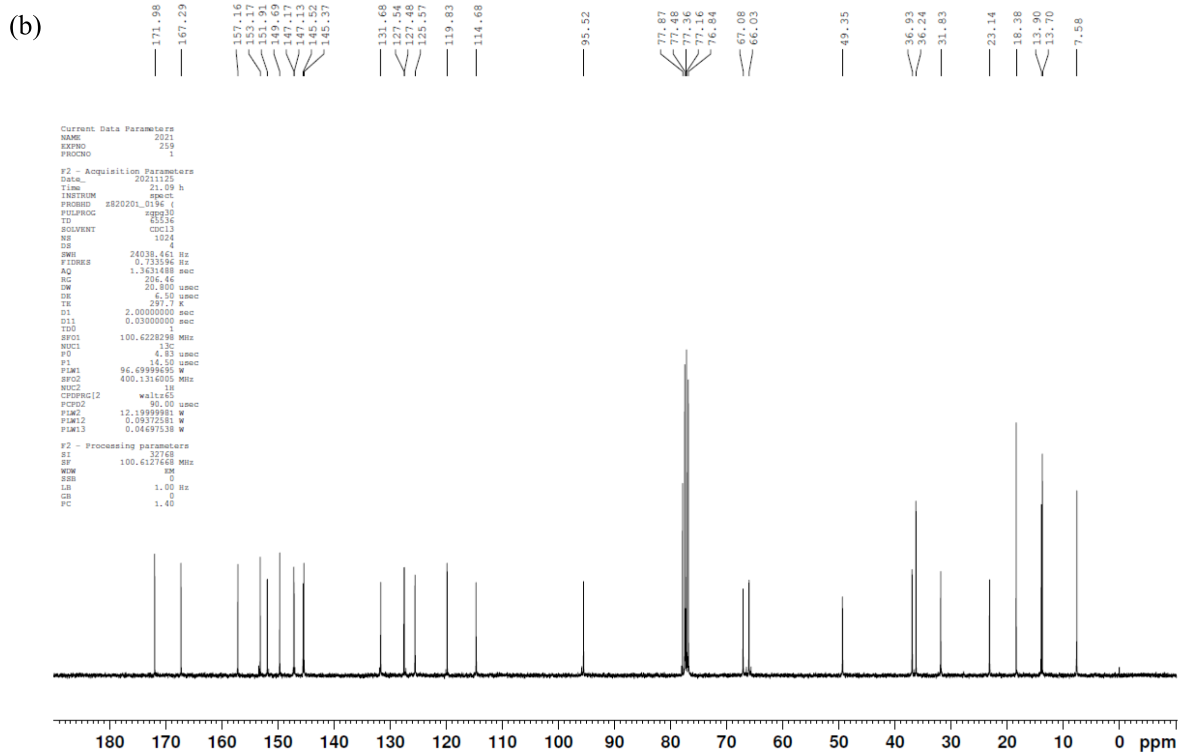
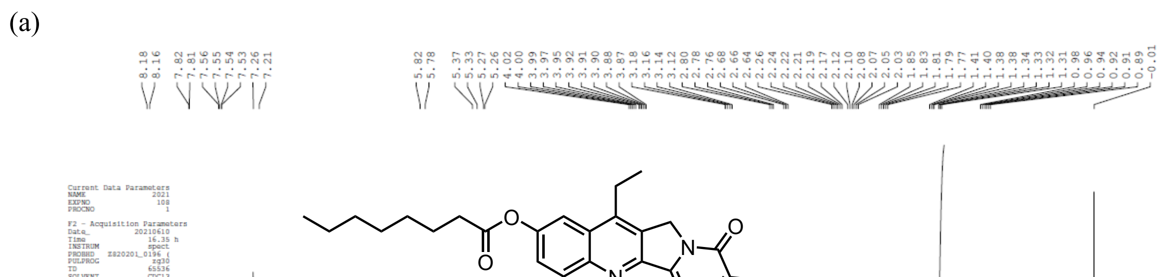


Figure S16. NMR spectrum of SNC4DC.

(a) ^{1}H NMR ($CDCl_3$, 400 MHz), (b) ^{13}C NMR ($CDCl_3$, 100 MHz).

(a)



(b)

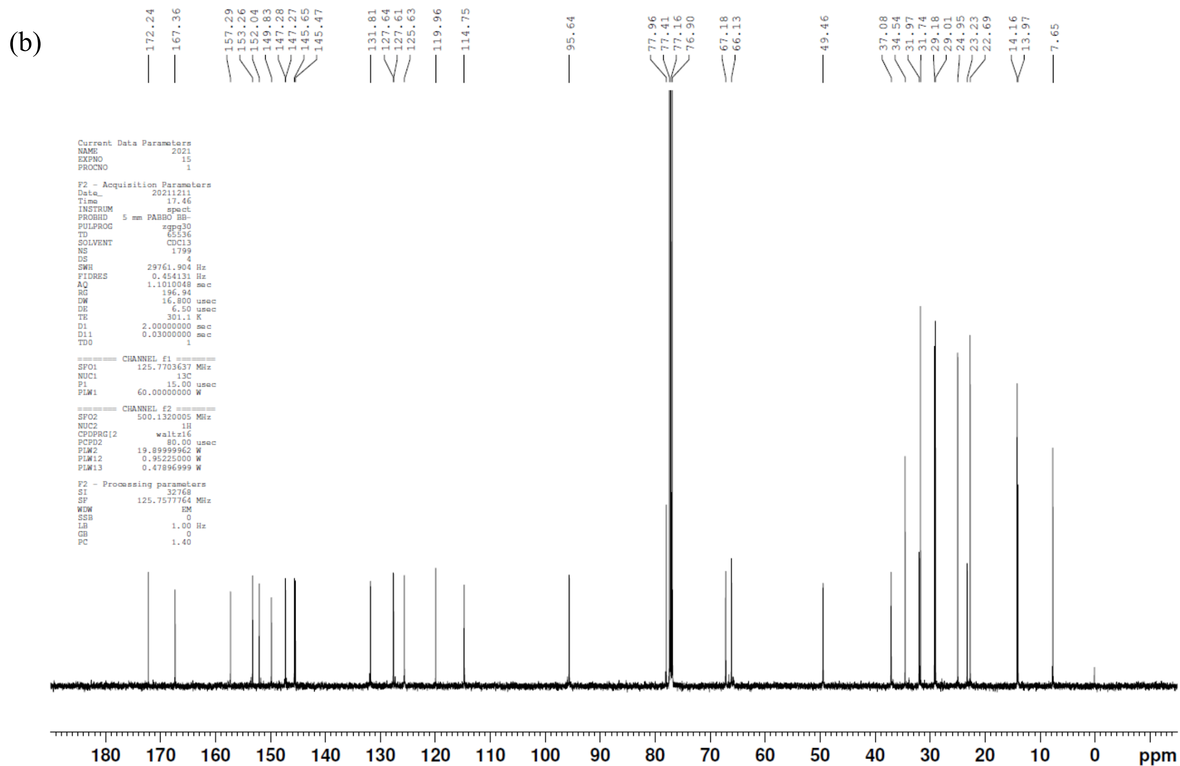
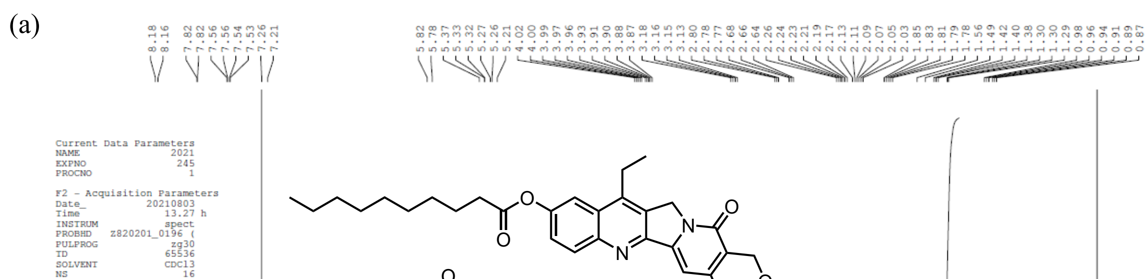


Figure S17. NMR spectrum of SNC8DC.

(a) ^1H NMR ($CDCl_3$, 400 MHz), (b) ^{13}C NMR ($CDCl_3$, 125 MHz).

(a)



(b)

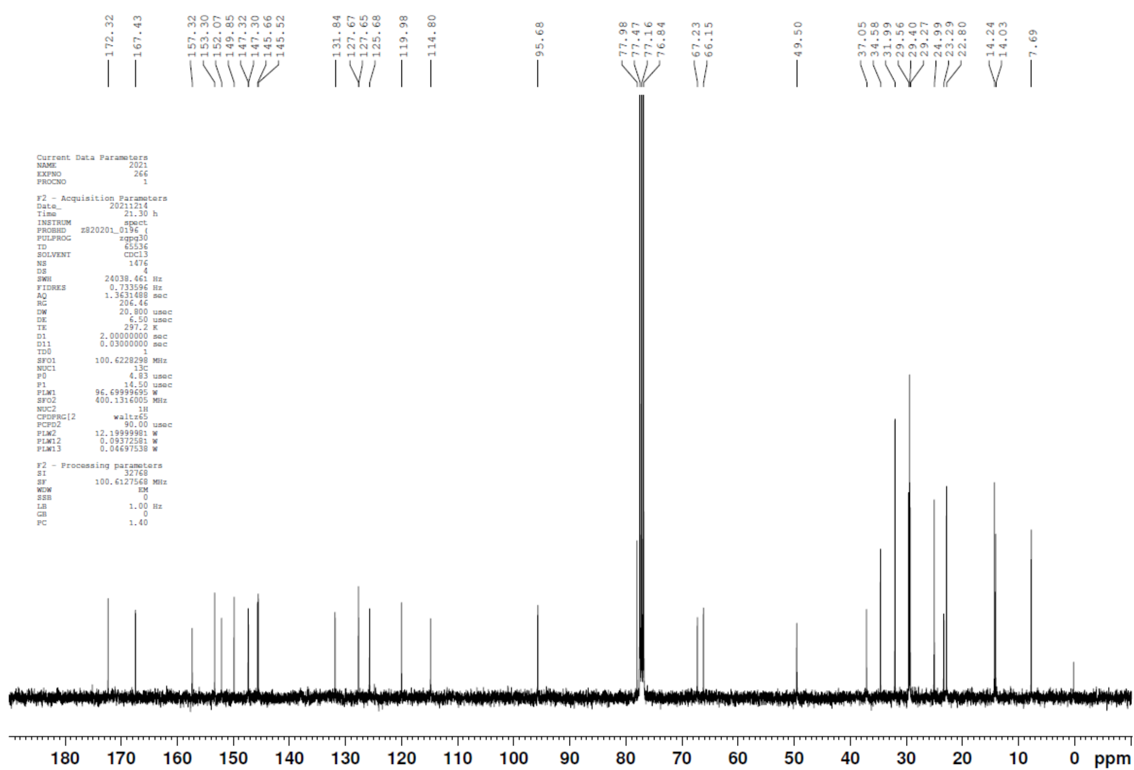


Figure S18. NMR spectrum of SNC10DC.

(a) ^{1}H NMR ($CDCl_3$, 400 MHz), (b) ^{13}C NMR ($CDCl_3$, 100 MHz).