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Supporting information for

Anticancer Nano-prodrugs with Drug Release triggered by Intracellular Dissolution and Hydrogen Peroxide Response

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Experimental Section

Materials.

Sodium dihydrogen phosphate (NaH_2PO_4), acetone, ammonia solution, dichloromethane (CH_2Cl_2), dimethyl sulfoxide (DMSO), hydrogen peroxide (H_2O_2), *N*,*N*-dimethylformamide (DMF), *tert*-butyl alcohol (*t*-BuOH), tetrahydrofuran (THF), acetic acid (AcOH), ammonium chloride (NH_4Cl), formic acid, sulfuric acid (H_2SO_4), 4-dimethylaminopyridine (DMAP), cesium carbonate (Cs_2CO_3), triethylamine (TEA), 5-sulfosalicylic acid (SSA) dihydrate, boron trifluoride diethyl ether complex ($BF_3 \cdot OEt_2$), potassium iodide (KI) were purchased from FUJIFILM Wako Pure Chemical Corporation. 2-Methyl-2-butene, camptothecin (CPT), pyridine-sulfur trioxide complex ($Py \cdot SO_3$) were purchased from Tokyo Chemical Industry Co., Ltd. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide Hydrochloride (EDC·HCl), chloroform ($CHCl_3$), diethyl ether (Et_2O), ethyl acetate (EtOAc), hexane, methanol (MeOH), hydrogen chloride (HCl), anhydrous magnesium sulfate ($MgSO_4$), celite 503RV, sodium chloride (NaCl) were purchased from NACALAI TESQUE, INC. Sodium chlorite ($NaClO_2$), esterase from porcine liver were purchased from Sigma-Aldrich Co.□ acetonitrile (MeCN), chloroform-*d* ($CDCl_3$), dimethyl sulfoxide-d_6 (DMSO-d_6), tetrahydrofuran-d_8 (THF-d_8), silica gel 60N (40–50 μm) for flash chromatography were purchased from KANTO CHEMICAL CO., INC. Silica gel plates 60F254 for thin layer

chromatography (TLC), PLC glass plate silica gel 60F254 was purchased from Merck. Dulbecco's modified eagle's medium (DMEM), phosphate buffered saline (PBS (−), no calcium, no magnesium), fetal bovine serum (FBS, South America origin), GlutaMax™ supplement, penicillin-streptomycin were purchased from ThermoFisher Scientific. Cell Counting Kit-8 was purchased from Dojindo Molecular Technologies, Inc. BIOXYTEC® H_2O_2-560™ Assay Kit was purchased from Funakoshi Co., Ltd. A549 human lung cancer cells, NHDF-Neo normal dermal fibroblast, neonatal cells were purchased from the RIKEN Cell Bank. All reagents were used without further purification.

Measurements.

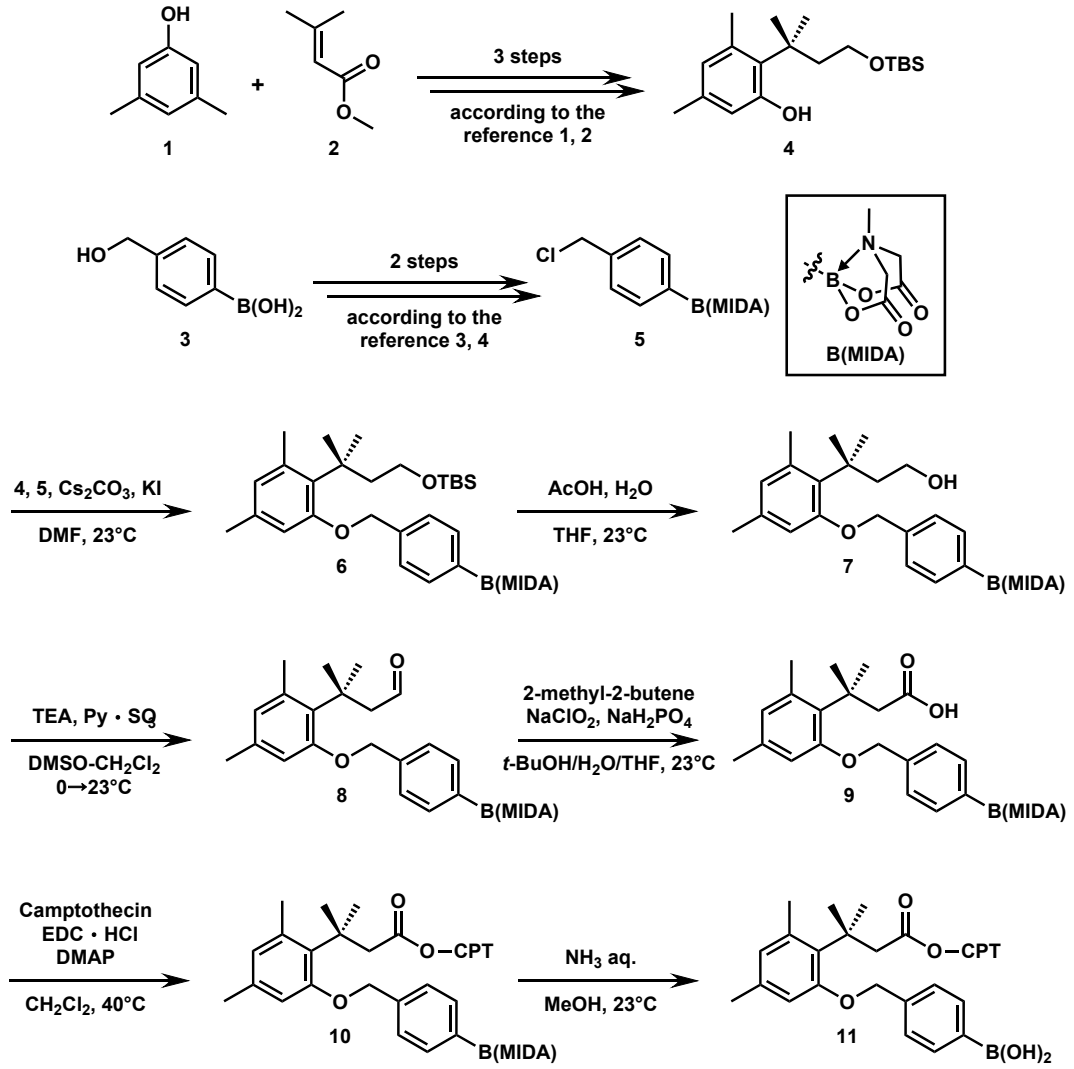
Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVANCE-400 and 500 spectrometer. High resolution mass spectrometry (HR-MS) was performed using a Bruker micro TOF-Q II-S1 by electrospray ionization time of flight (ESI-TOF) reflection experiment. Optical rotations were recorded on an Anton Paar MCP 100 modular circular polarimeter. Size distribution and zeta potential were measured using a Malvern Zetasizer NanoZS. Scanning electron microscope (SEM) images were observed using Hitachi S-4800. Powder X-ray power diffraction (PXRD) analysis was performed using RIGAKU RINT2200. Cell viability and intracellular H_2O_2 assay were evaluated using Bio-Rad iMark microplate reader. Centrifugation steps was performed using TOMY MDX-310 High Speed Refrigerated Micro Centrifuges. High-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) was performed using a LCMS-8030 manufactured by Shimadzu Corporation. Melting points (Mp) were determined using a METTLER TOLEDO Melting Point System MP80.

General Method of Organic Synthesis

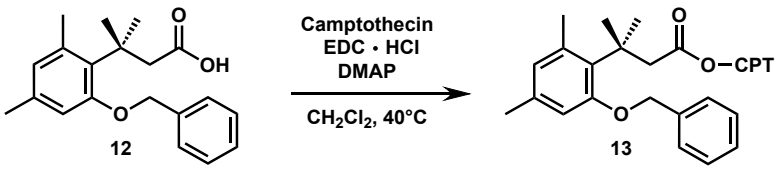
All air- or moisture-sensitive reactions were carried out in glassware under an argon atmosphere. Reactions were monitored by analytical TLC carried out on 0.25-mm silica gel plates. Visualization of the developed plate was performed using UV absorbance at 254 nm or 365 nm and ceric ammonium molybdate solution. Flash chromatography was performed on silica gel 60N (40–50 μm) with the indicated solvent systems. ^{1}H and ^{13}C 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, THF-d_8 at δ 1.72 ppm for ^{1}H, and δ 25.31 ppm for ^{13}C NMR. ^{11}B NMR spectra were calibrate using $BF_3 \cdot OEt_2$ added to the deuterated solvent as an international reference; 0.0 ppm. The following abbreviations were used to explain NMR peak multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet.

Synthetic procedures of TML group and CPT-TML

Compound (**4**)[1,2], compound (**5**)[3,4], and compound (**12**)[5], were synthesized according to the reference.

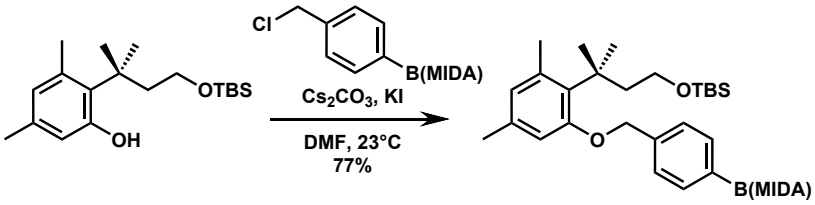


Scheme S1. Synthetic scheme of compound (**11**) (**CPT-TML-ArB(OH)$_2$**).



Scheme S2. Synthetic scheme of compound (**13**) (**CPT-TML-Ar**).

Synthesis of 2-(4-((2-(4-((tert-butyldimethylsilyl)oxy)-2-methylbutan-2-yl)-3,5-dimethylphenoxy)methyl)phenyl)-6-methyl-1,3,6,2-dioxazaborocane-4,8-dionecompound (6):

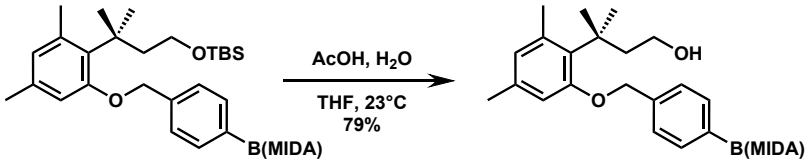


Scheme S3. Synthesis of compound (**6**).

2-(4-((tert-butyldimethylsilyl)oxy)-2-methylbutan-2-yl)-3,5-dimethylphenol (compound (**4**)) (2.65 g, 8.23 mmol), 2-(4-(chloromethyl)phenyl)-6-methyl-1,3,6,2-dioxazaborocane-4,8-dione (compound (**5**)), Cs_2CO_3 (6.57 g, 20.2 mmol), KI (3.38 g, 20.3 mmol) was added anhydrous DMF (82 mL, 0.1 M). After stirring for 3 d at 23°C, the reaction mixture was quenched with saturated aqueous solution of NH_4Cl and extracted with EtOAc. The organic layer was washed with H_2O and brine. The organic layer then dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (Hexane/EtOAc = 100/0–1/6) to give 2-(4-((2-(4-((tert-butyldimethylsilyl)oxy)-2-methylbutan-2-yl)-3,5-dimethylphenoxy)methyl)phenyl)-6-methyl-1,3,6,2-dioxazaborocane-4,8-dione (compound (**6**)) (3.62 g, 6.37 mmol, 77%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.55 (2H, d, J = 8.1 Hz), 7.48 (2H, d, J = 8.1 Hz), 6.63 (1H, s), 6.54 (1H, s), 5.04 (2H, s), 3.92 (2H, d, J = 16.3 Hz), 3.78 (2H, d, J = 16.3 Hz), 3.47 (2H, t, J = 7.67 Hz), 2.58

(3H, s), 2.49 (3H, s), 2.23 (3H, s), 2.12 (2H, t, J = 7.7 Hz), 1.50 (6H, s), 0.81 (9H, s), −0.071 (6H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 167.8, 158.7, 139.5, 137.8, 135.9, 132.6, 131.5, 127.7, 127.6, 112.5, 70.9, 61.9, 61.6, 47.7, 45.7, 40.0, 32.4, 26.1, 26.0, 20.9, 18.4, −5.13. The carbon directly attached to the boron atom was not detected due to quadrupolar broadening. ^{11}B NMR (160 MHz, $CDCl_3$): δ = 14.4. HR-MS (ESI-TOF): *m/z* calculated for $C_{31}H_{47}BNO_6Si$ ($[M + H]^+$) 568.3265, found 568.3265. Mp: 120.0°C.

Synthesis of 2-(4-((2-(4-hydroxy-2-methylbutan-2-yl)-3,5-dimethylphenoxy)methyl) phenyl)-6-methyl-1,3,6,2-dioxazaborocane-4,8-dione (7):

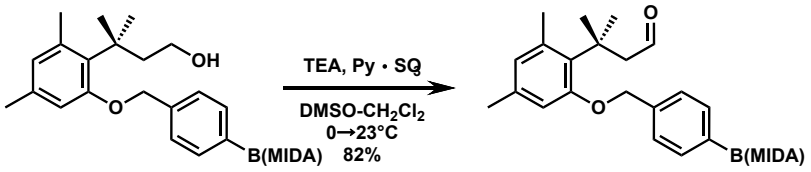


Scheme S4. Synthesis of compound (**7**).

Compound (**6**) (1.21 g, 2.67 mmol), AcOH (105 mL), H_2O (21.0 mL) was added anhydrous THF (21.0 mL) (AcOH/H_2O/THF = 5/1/1, 147 mL, 0.1 M). After stirring for 15 h at 23°C, the reaction mixture was quenched with H_2O and extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (Hexane/EtOAc = 1/10) to 2-(4-((2-(4-hydroxy-2-methylbutan-2-yl)-3,5-dimethylphenoxy)methyl)phenyl)-6-methyl-1,3,6,2-dioxazaborocane-4,8-dione (compound (**7**)) (766 mg, 1.69 mmol, 79%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.56 (2H, d, J = 8.1 Hz), 7.49 (2H, d, J = 8.1 Hz), 6.66 (1H, s), 6.57 (1H, s), 5.07 (2H, s), 3.91 (2H, d, J = 16.3 Hz), 3.79 (2H, d, J = 16.3 Hz), 3.51 (2H, t, J = 7.1 Hz), 2.59 (3H, s), 2.50 (3H, s), 2.24 (3H, s), 2.14 (2H, t, J = 7.3 Hz), 1.51 (6H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 168.5, 158.6, 139.1, 137.8, 136.2, 132.7, 131.3, 127.8, 127.7, 112.6, 77.4, 70.9, 62.0, 61.2, 47.8, 45.6, 39.8, 32.3, 26.0, 20.9. The carbon directly attached to the boron atom was not detected due to quadrupolar broadening. ^{11}B NMR (160 MHz,

$CDCl_3$): δ = 12.4. HR-MS (ESI-TOF): *m/z* calculated for $C_{25}H_{32}BNNaO_6$ ($[M + Na]^+$) 476.2240, found 476.2237. Mp: 112.2°C.

Synthesis of 3-(2,4-dimethyl-6-((4-(6-methyl-4,8-dioxo-1,3,6,2-dioxazaborocan-2-yl)benzyl)oxy)phenyl)-3-methylbutanal (8):

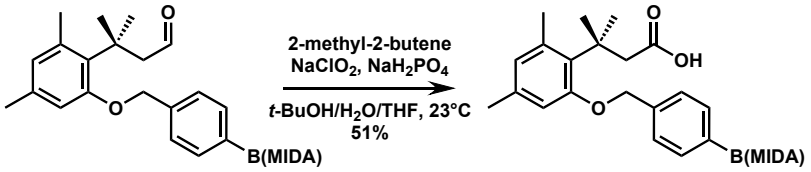


Scheme S5. Synthesis of compound (**8**).

Compound (**7**) (195 mg, 0.430 mmol), DMSO (4.4 mL), TEA (0.250 mL, 1.79 mmol), $Py{\cdot}SO_3$ (154 mg, 0.965 mmol) was added anhydrous CH_2Cl_2 (4.4 mL) (CH_2Cl_2/DMSO = 1/1, 8.8 mL, 0.05 M) at 0°C. After stirring for 12 h at 23°C, the reaction mixture was quenched with H_2O and extracted with $CHCl_3$. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (Hexane/EtOAc = 100/0–1/10) to give 3-(2,4-dimethyl-6-((4-(6-methyl-4,8-dioxo-1,3,6,2-dioxazaborocan-2-yl)benzyl)oxy)phenyl)-3-methylbutanal (compound (**8**)) (158 mg, 0.350 mmol, 82 %) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.45 (1H, t, J = 2.7 Hz), 7.56 (2H, d, J = 8.0 Hz), 7.45 (2H, d, J = 8.0 Hz), 6.69 (1H, s), 6.60 (1H, s), 5.07 (2H, s), 3.92 (2H, d, J = 16.3 Hz), 3.79 (2H, d, J = 16.3 Hz), 2.89 (2H, d, J = 2.7 Hz), 2.60 (3H, s), 2.52 (3H, s), 2.24 (3H, s), 1.58 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 204.7, 168.1, 157.9, 138.8, 137.6, 136.8, 132.8, 129.7, 128.0, 127.8, 112.5, 70.8, 62.0, 56.7, 47.8, 38.9, 32.0, 31.7, 25.9, 22.8, 20.9, 14.3. The carbon directly attached to the boron atom was not detected

due to quadrupolar broadening. ^{11}B NMR (160 MHz, $CDCl_3$): δ = 11.9. HR-MS (ESI-TOF): *m/z* calculated for $C_{25}H_{31}BNO_6$ ($[M + H]^+$) 452.2243, found 452.2253. Mp: 114.2°C.

Synthesis of 3-(2,4-dimethyl-6-((4-(6-methyl-4,8-dioxo-1,3,6,2-dioxazaborocan-2-yl)benzyl)oxy)phenyl)-3-methylbutanoic acid (9):

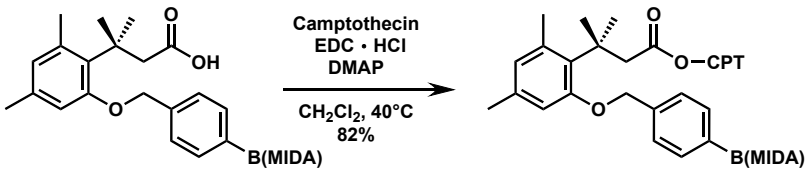


Scheme S6. Synthesis of compound (**9**).

Compound (**8**) (72.8 mg, 0.161 mmol), *t*-BuOH (1.5 mL), 2-methyl-2-butene (0.165 mL, 1.56 mmol) was added anhydrous THF (6.0 mL) at 23 °C. Then, a solution of $NaClO_2$ (26.3 mg, 0.291 mmol) and NaH_2PO_4 (47.0 mg, 0.392 mmol) in H_2O (1.5 mL) dropwise (THF/*t*-BuOH/H_2O = 4/1/1, 9.0 mL, 0.017M). After stirring for 1 h at 23°C, the reaction mixture was quenched with saturated aqueous solution of NH_4Cl and extracted with EtOAc. The organic layer was washed with H_2O and brine. The organic layer then dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (Hexane/EtOAc = 100/0–1/10) to give 3-(2,4-dimethyl-6-((4-(6-methyl-4,8-dioxo-1,3,6,2-dioxazaborocan-2-yl)benzyl)oxy) phenyl)-3-methylbutanoic acid (compound (**9**)) (38.8 mg, 83.0 μmol, 51%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 11.6 (1H, s), 7.46 (4H, s), 6.76 (1H, s), 6.49 (1H, s), 5.07 (2H, s), 4.35 (2H, d, J = 17.2 Hz), 4.13 (2H, d, J = 17.2 Hz), 2.83 (2H, s), 2.45 (3H, s), 2.16 (3H, s), 1.53 (6H, s). The methyl proton directly attached to the nitrogen atom was not detected due to overlaps with peak of DMSO. ^{13}C NMR (100 MHz, DMSO-

d_6): δ = 173.4, 169.4, 157.9, 138.0, 136.9, 135.1, 132.6, 130.3, 127.1, 126.8, 112.6, 70.2, 61.8, 47.6, 46.5, 31.6, 25.3, 20.4. The carbon around 40 ppm was not detected due to overlaps with peak of DMSO. The carbon directly attached to the boron atom was not detected due to quadrupolar broadening. ^{11}B NMR (160 MHz, DMSO-d_6): δ = 14.2. HR-MS (ESI-TOF): *m/z* calculated for $C_{25}H_{30}BNNaO_7$ ($[M + Na]^+$) 490.2011, found 490.2024. Mp: 200.2°C.

Synthesis of (S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7] indolizino[1,2-b]quinolin-4-yl 3-(2,4-dimethyl-6-((4-(6-methyl-4,8-dioxo-1,3,6,2-dioxazaborocan-2-yl)benzyl)oxy)phenyl)-3-methylbutanoate (10):

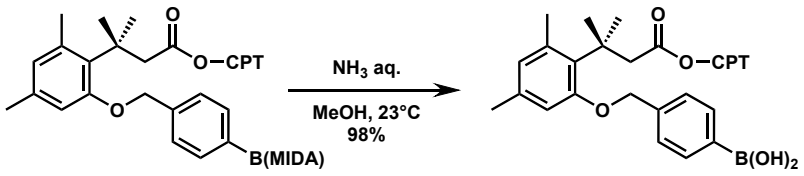


Scheme S7. Synthesis of compound (**10**).

Compound (**9**) (66.4 mg, 0.142 mmol), camptothecin (50.9 mg, 0.146 mmol), EDC·HCl (110 mg, 0.573 mmol), DMAP (68.5 mg, 0.561 mmol) was added anhydrous CH_2Cl_2 (14.2 mL, 0.01 M). After stirring for 12 h at 40°C, the reaction mixture quenched with saturated aqueous solution of NH_4Cl and extracted with $CHCl_3$. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (EtOAc/MeOH/Acetone = 8/1/1 then Et_2O/MeOH = 10/1) to give (S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl 3-(2,4-dimethyl -6-((4-(6-methyl-4,8-dioxo-1,3,6,2-dioxazaborocan-2-yl)benzyl)oxy)phenyl)-3-methyl butanoate (compound (**10**)) (92.7 mg, 0.116 mmol, 82%) as a pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.39 (1H, s), 8.20 (1H, d, J = 8.1 Hz), 7.96 (1H, d, J = 8.2 Hz), 7.84 (1H, ddd, J = 8.5, 7.0, 1.5 Hz), 7.68 (1H, ddd, J = 8.1, 6.9, 1.1 Hz), 7.54–7.49 (4H, m), 7.02 (1H, s), 6.69 (1H, s), 6.51 (1H, s), 5.59 (1H, d, J = 17.2 Hz), 5.34 (1H, d, J =

17.2 Hz), 5.28–5.25 (3H, m), 5.15 (1H, d, J = 11.4 Hz), 3.89–3.80 (3H, m), 3.74 (1H, d, J = 16.4 Hz), 3.15 (2H, q, J = 14.1 Hz), 2.54 (3H, s), 2.50 (3H, s), 2.11–2.06 (1H, m), 2.02 (3H, s), 1.98–1.91 (1H, m), 1.58 (6H, s), 0.82 (3H, t, J = 7.5 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.8, 167.9, 167.4, 167.3, 158.1, 157.5, 152.6, 149.0, 146.2, 146.1, 139.1, 137.6, 136.1, 132.7, 131.3, 130.8, 130.5, 129.6, 128.6, 128.5, 128.3, 128.1, 128.0, 127.9, 120.2, 112.4, 96.5, 75.4, 70.9, 67.1, 61.9, 50.0, 47.5, 47.1, 40.0, 31.7, 31.6, 31.2, 29.8, 25.9, 20.8, 7.61. The carbon directly attached to the boron atom was not detected due to quadrupolar broadening. ^{11}B NMR (160 MHz, $CDCl_3$): δ = 12.8. HR-MS (ESI-TOF): *m/z* calculated for $C_{45}H_{45}BN_3O_{10}$ ($[M + H]^+$) 798.3200, found 798.3216. $[\alpha]_D^{25}$ = −34 (c = 0.10 in $CHCl_3$). Mp: 185.6°C.

Synthesis of (S)-(4-((2-(4-((4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano [3',4':6,7]indolizino[1,2-b]quinolin-4-yl)oxy)-2-methyl-4-oxobutan-2-yl)-3,5-dimethyl phenoxy)methyl)phenyl)boronic acid (11):

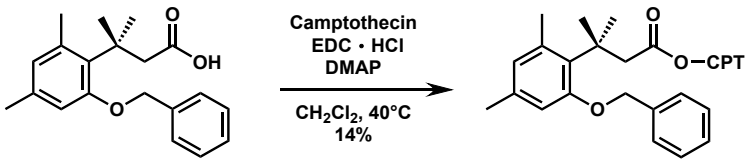


Scheme S8. Synthesis of compound (**11**).

Compound (**10**) (53.7 mg, 67.3 μmol) in MeOH (62.5 mL, 1.0 mM) was added ammonia solution (94 μL, 125 μmol). After stirring for 15 h at 23°C, the reaction mixture was quenched with saturated aqueous solution of NH_4Cl and extracted with EtOAc. The organic layer was washed with H_2O and brine, dried over $MgSO_4$, filtered. The isolated solution was concentrated under reduced pressure to give (S)-(4-((2-(4-((4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)oxy)-2-methyl-4-oxobutan-2-yl)-3,5-dimethylphenoxy)methyl)phenyl)boronic acid (compound (**11**)) (45.1 mg, 65.7 μmol, 98%) as a pale yellow solid. ^{1}H NMR (400 MHz, THF-d_8): δ = 8.51 (1H, s), 8.22 (1H, d, J = 8.6 Hz), 8.00 (1H, d, J = 3.1 Hz), 7.83–7.77 (3H, m), 7.64 (1H, ddd, J = 8.1, 6.9, 1.2 Hz), 7.54 (2H, d, J = 8.1 Hz), 7.12 (2H, s), 6.92 (1H, s), 6.74 (1H, s), 6.40 (1H, s), 5.43–5.37 (2H, m), 5.27–5.17 (4H, m), 3.23 (1H, d, J = 15.8 Hz), 3.04 (1H, d, J = 15.8 Hz), 2.49 (3H, s), 2.06–1.97 (1H, m), 1.94–1.85 (4H, m, overlaps with peak of EtOAc), 1.57 (3H, s), 1.52 (3H, s), 0.76 (3H, t, J = 7.5 Hz). ^{13}C NMR (100

MHz, THF-d_8): δ = 171.6, 167.6, 159.2, 157.7, 153.9, 149.7, 146.9, 146.8, 140.3, 138.0, 136.1, 135.2, 131.8, 131.2, 130.9, 130.6, 130.3, 129.3, 129.2, 128.2, 128.1, 128.0, 120.9, 113.2, 96.2, 76.1, 72.0, 50.7, 48.0, 40.6, 32.3, 32.0, 31.9, 30.6, 26.1, 20.6, 7.87. The carbon directly attached to the boron atom was not detected due to quadrupolar broadening. ^{11}B NMR (160 MHz, THF-d_8): δ = 21.0. HR-MS (ESI-TOF): *m/z* calculated for $C_{40}H_{40}BN_2O_8$ ($[M + H]^+$) 687.2879, found 687.2868. $[\alpha]_D^{25} = -40$ (*c* = 0.10 in THF). Mp: 192.8°C.

Synthesis of (S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7] indolizino[1,2-b]quinolin-4-yl 3-(2-(benzyloxy)-4,6-dimethylphenyl)-3-methyl butanoate (13):



Scheme S9. Synthesis of compound (**13**).

3-(2-(benzyloxy)-4,6-dimethylphenyl)-3-methylbutanoic acid (compound (**12**)) (184 mg, 0.588 mmol), camptothecin (201 mg, 0.578 mmol), EDC·HCl (447 mg, 2.33 mmol), DMAP (216 mg, 1.77 mmol) was added anhydrous CH_2Cl_2 (57 mL, 0.01 M) was added. After stirring for 24 h at 40°C, the reaction mixture was filtrated by celite 503RV. The filtrate was quenched with saturated aqueous solution of NH_4Cl and extracted with $CHCl_3$. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (Hexane/Acetone = 1/2) and preparative TLC (CH_2Cl_2/MeOH/Hexane = 8/1/1) to give (S)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl 3-(2-(benzyloxy)-4,6-dimethylphenyl)-3-methylbutanoate (compound (**13**)) (51.3 mg, 79.8 μmol, 14%) as a pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.39 (1H, s), 8.23 (1H, d, J = 8.4 Hz), 7.96 (1H, d, J = 7.1 Hz), 7.84 (1H, ddd, J = 8.5, 6.9, 1.5 Hz), 7.68 (1H, ddd, J = 8.1, 6.9, 1.2 Hz), 7.55 (2H,

d, J = 7.1 Hz), 7.40 (2H, t, J = 7.3 Hz), 7.32 (1H, d, J = 7.3 Hz), 7.03 (1H, s), 6.68 (1H, s), 6.48 (1H, s) 5.59 (1H, d, J = 17.2 Hz), 5.36–5.30 (2H, m), 5.25–5.19 (2H, m), 5.14 (1H, d, J = 11.2 Hz), 3.25 (1H, d, J = 15.8 Hz), 3.10 (1H, d, J = 15.8 Hz), 2.50 (3H, s), 2.10–2.01 (1H, m), 1.96 (3H, s), 1.94–1.88 (1H, m), 1.61 (3H, s), 1.57 (3H, s), 0.81 (3H, t, J = 7.5 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.8, 167.8, 158.1, 157.5, 152.7, 149.0, 146.3, 145.9, 137.7, 137.5, 135.8, 131.2, 130.8, 130.4, 129.7, 128.8, 128.6, 128.4, 128.3, 128.2, 128.1, 128.0, 127.7, 120.3, 112.3, 96.7, 75.2, 71.2, 67.0, 50.0, 47.5, 39.9, 31.9, 31.7, 31.5, 25.9, 20.6, 7.58. HR-MS (ESI-TOF): *m/z* calculated for $C_{40}H_{39}N_2O_6$ ([M + H]$^+$) 643.2802, found 643.2789. $[\alpha]_D^{25}$ = −63 (c = 0.10 in $CHCl_3$). Mp: 219.1°C.

Fabrication of CPT-TML nano-prodrugs

The **CPT-TML** nano-prodrugs (**NPDs**) were fabricated by the reprecipitation method.[6] Deionized water was purified to 18.2 MΩcm using an Arium 611UV (Sartorius Mechtronics Japan K.K.). 9.9 mL of deionized water was transferred to screw tube and stirred at 1,500 rpm at 23°C. Then, the **CPT-TML** (10 mM) was dissolved in THF. 100 μL of the **CPT-TML** THF solution was rapidly injected into vigorously stirred 9.9 mL of deionized water by a microsyringe.

DLS measurements

0.75 mL of the **NPDs** (0.1 mM) was added to the capillary cell. For the measurement He-Ne laser (3.0 mW, 633 nm) was used and the temperature was set at 25°C.

SEM sampling

The **NPDs** were fixed and dried onto Whatman® Nuclepore Track-Etched Membranes (ϕ = 0.05 μm) using diaphragm pump and let dried overnight in desiccator. Dried sample was attached to specimen mount using carbon conductive adhesive tapes. The sample was sputtering with platinum prior to SEM measurements.

Gaussian curve fitting process

A histogram and a related gaussian-fitting curve of the size distribution of the **NPDs** in the SEM images were calculated by manual counting more than 300 nanoparticles. ImageJ (NIH, Bethesda, MD, USA) was used for manual counting.

PXRD sample preparation

Dispersion solution of the **NPDs** was filtered and dried onto Whatman® Nuclepore Track-Etched Membranes (ϕ = 0.05 μm) using diaphragm pump. The samples were dried for overnight prior to the PXRD measurement.

Cell viability assay

The A549 human lung cancer cells complete medium was DMEM supplemented with 10% FBS, 1% penicillin-streptomycin and 1% GlutaMax™; the NHDF-Neo normal dermal fibroblast, neonatal cells was DMEM supplemented with 5% FBS and 1% penicillin-streptomycin. All cells were incubated aseptically in a 37°C incubator containing 5% CO_2. The cultured cells were seeded in 96-well plates (1×10^4 cells/well) and incubated for 24 h. Then, the culture medium was removed, and 100 μL of medium containing nano-prodrugs were added. After 48 h at 37°C, the number of viable cells were evaluated using the Cell Counting Kit-8 and a Microplate Reader. Cell viabilities were normalized to OD_{450}–OD_{620} for the untreated cells. Assays were performed in triplicate.

Drug release kinetics in CPT-TML NPDs (H_2O_2 solution, PBS (−))

The 300 μL of H_2O_2 solution (200 μM) and 300 μL of **CPT-TML NPDs** dispersion (20 μM) was mixed. The mixture was incubated at 37°C in a water bath and 300 μL of samples were withdrawn at different time-points (0, 1, 6, 24 and 48 h). The samples were extracted with 300 μL of DMSO. 200 μL of the extracted solvent was diluted with 800 μL of acidified extraction solvent (MeCN/MeOH = 1/1 acidified with 3% glacial acetic acid). The extracted solution was analyzed by HPLC-MS/MS.

For comparison, a **CPT-TML NPDs** dispersion (20 μM) was treated with PBS (−) instead of H_2O_2 solution and drug release kinetics were evaluated in the same manner.

Drug release kinetics in CPT-TML DMSO solution (H_2O_2 solution, PBS (−))

The 300 μL of H_2O_2 solution (200 μM), and 300 μL of **CPT-TML** DMSO solution (20 μM) was added. The mixture was incubated at 37°C in a water bath and 100 μL of samples were withdrawn at different time-points (0, 1, 6, 24 and 48 h). The samples were extracted with 900 μL of acidified extraction solvent (MeCN/MeOH = 1/1 acidified with 3% glacial acetic acid). The extracted solution was analyzed by HPLC-MS/MS.

For comparison, a **CPT-TML** DMSO solution (20 μM) was treated with PBS (−) instead of H_2O_2 solution and drug release kinetics were evaluated in the same manner.

Drug release kinetics in CPT-TML NPDs (esterase from porcine liver)

The 300 μL of 20 units or 200 units esterase in PBS (−), and 300 μL of **CPT-TML NPDs** dispersion (20 μM) was added. The mixture was incubated at 37 °C in a water bath at different time-points (0 and 48 h). The samples were mixed with 600 μL of DMSO for esterase inactivation. After precipitating the inactivated esterase by centrifugation (15,000 × g, 15 min, 4°C), 200 μL of the supernatant was diluted with 800 μL of acidified extraction solvent (MeCN/MeOH = 1/1 acidified with 3% glacial acetic acid). The extracted solution was analyzed by HPLC-MS/MS.

Calibration standard solutions for quantification of drug release kinetics in CPT-TML

Two types of the **CPT-TML-ArB(OH)$_2$** standard solutions derived from **CPT-TML-ArB(OH)$_2$ NPDs** or **CPT-TML-ArB(OH)$_2$** DMSO solution were prepared. These standard solutions were prepared from 2,000 nM **CPT-TML-ArB(OH)$_2$ NPDs** or **CPT-TML-ArB(OH)$_2$** DMSO solution, serially diluted with MeCN. The calibration curves were obtained by standard solutions at concentrations of 10–1,000 nM (Fig. S6A–S6B).

Two types of the **CPT-TML-Ar** standard solutions derived from **CPT-TML-Ar NPDs** or **CPT-TML-Ar** DMSO solution were prepared in the above same manner. The calibration curves were obtained by standard solutions at concentrations of 10–1,000 nM (Fig. S6C–S6D).

To monitor the **CPT** release from **CPT-TML NPDs** or **CPT-TML** DMSO solution, the **CPT** standard solutions derived from **CPT** DMSO solution were prepared in the above same manner. The calibration curves were obtained by standard solutions at concentrations of 10–1,000 nM (Fig. S6E).

HPLC-MS/MS conditions

The HPLC conditions for all samples were as follows: column, reverse-phase column (Imtakt Unison UK-C8, 3 μm, ϕ2 × 100 mm); column temperature, 40 ℃; mobile phase, gradient elution from water with 0.1% formic acid/MeCN with 0.1% formic acid (v/v) = 60/40 linearly increased 10/90 for 10 min; flow rate 0.3 mL/min; injection volume, 1 μL; MS/MS was performed in $[M + H]^+$ as the precursor ions and the major fragment ions from $[M + H]^+$ were detected.

Parameters and transitions in the determination method for HPLC-MS/MS conditions

All compounds were monitored by tandem mass spectrometry in electrospray-positive ionization and multiple reaction mode programmed to the following parameters.

Camptothecin (CPT): Retention time = 1.6 min, Q1 pre bias = −27.0 V, Collision energy = −23.6 eV, Q3 pre bias = −30.0 V, Transition = 349.00 → 305.35 *m/z*.

CPT-TML-ArB(OH)$_2$: Retention time = 5.5 min, Q1 pre bias = −36.0 V, Collision energy = −16.0 eV, Q3 pre bias = −22.0 V, Transition = 687.00 → 431.05 *m/z*.

CPT-TML-Ar: Retention time = 7.7 min, Q1 pre bias = −34.0 V, Collision energy = −14.0 eV, Q3 pre bias = −15.0 V, Transition = 643.00 → 431.10 *m/z*.

CPT-TML-ArOH: Retention time = 6.1 min, Q1 pre bias = −36.0 V, Collision energy = −18.0 eV, Q3 pre bias = −15.0 V, Transition = 659.00 → 431.20 *m/z*.

lactonized TML group: Retention time = 2.5 min, Q1 pre bias = −22.0 V, Collision energy = −8.0 eV, Q3 pre bias = −15.0 V, Transition = 205.10 → 87.10.

Intracellular H_2O_2 assay

The intracellular H_2O_2 concentration were quantified using the BIOXYTEC® H_2O_2-560™ Assay Kit (R1: 25 mM ammonium iron (II) sulfate, 2.5 M H_2SO_4, R2: 100 mM sorbitol, 125 μM xylenol orange in water). The A549 human lung cancer cells or NHDF-Neo normal dermal fibroblast, neonatal cells were collected, cell numbers counted (> 3.8 × 10^7 cells). Cells dispersion in medium were centrifugated at 200 × g for 10 min at 4°C. After discard the supernatant, the precipitated cells were washed with 1000 μL of PBS (−). Cells dispersion in PBS (−) were centrifugated at 200 × g for 10 min at 4°C. After discard the supernatant, 400 μL of 10 mM HCl was added to the pellets and freezed twice to lyse cells (freeze process: 10 min at −80°C, defrost process: 2–3 min at 37°C). After added 400 μL of 5% SSA, the lysate was centrifuged at 8000 × g for 10 min at 4°C, and transfer 300 μL of supernatant to new tube. Then, 300 μL of deionized water was added to the 300 μL of supernatant containing 1% SSA. The 20 μL of extracted solution was added 200 μL of working reagent (R1/R2 = 1/100) or blank reagent (2.5 M H_2SO_4/R2 = 1/100). After 30 min at room temperature, intracellular H_2O_2 concentration were evaluated using a Microplate Reader by (OD_{560} incubated with working reagent) – (OD_{560} incubated with the blank reagent). Assays were performed in triplicate.

Calibration standard solutions for quantification of intracellular H_2O_2

The standard solutions were prepared from 100 μM H_2O_2 solution, serially diluted with medium. The calibration curves were obtained using standard solutions at concentrations of 1.57–50 μM. The calibration curves were calculated according to the quantification of intracellular H_2O_2 assay protocol.

Supporting Figures

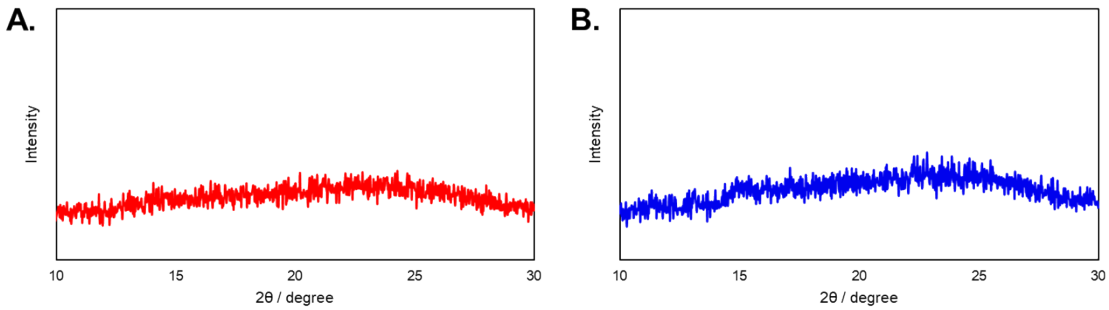


Fig. S1. PXRD patterns of (A) **CPT-TML-ArB(OH)$_2$ NPDs** and (B) **CPT-TML-Ar NPDs**.

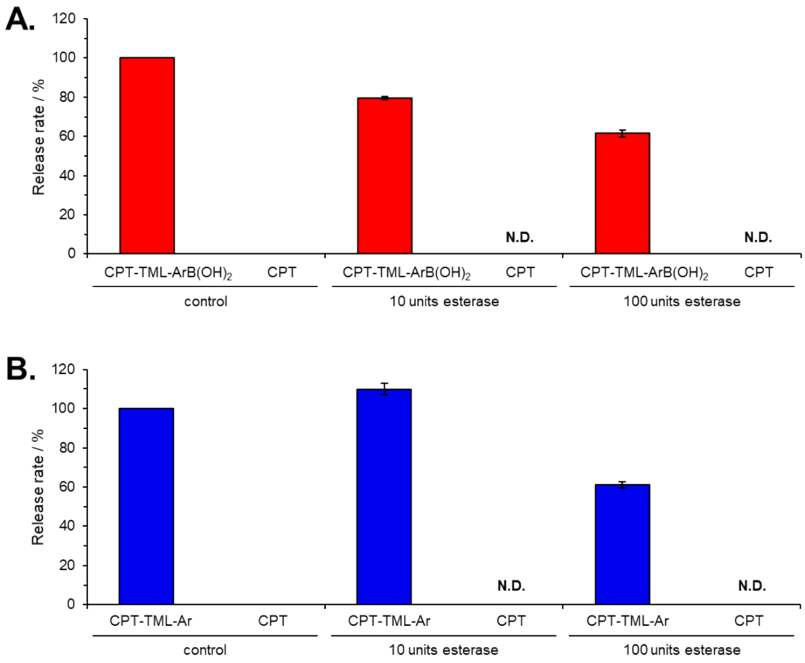


Fig. S2. Release rate of (A) **CPT-TML-ArB(OH)$_2$ NPDs** and (B) **CPT-TML-Ar NPDs** in water incubated with 10 units or 100 units esterase in PBS (−) at 37°C for 48 h. The relative amounts were calculated based on the concentration of the **CPT-TML NPDs** before incubation as the control. These results are indicated as the mean ± standard error (n = 3). N.D.: not detected.

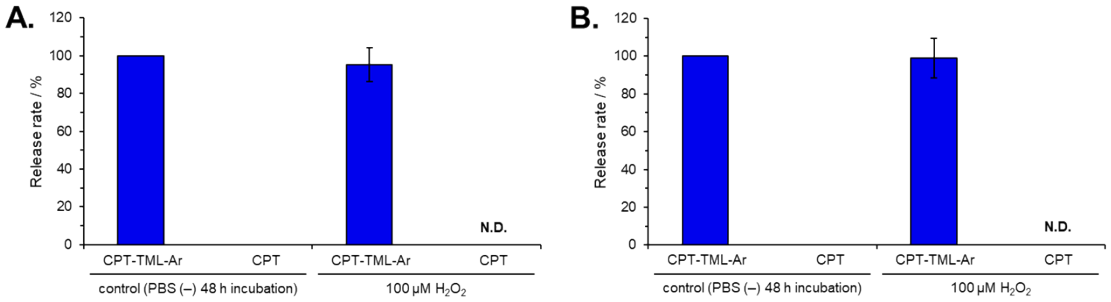


Fig. S3. Release rate of (A) **CPT-TML-Ar NPDs** in water and (B) **CPT-TML-Ar** in DMSO incubated with 100 μM H_2O_2 solution at 37°C for 48 h. The relative amounts were calculated based on the concentration of the **CPT-TML-Ar** after incubation in PBS (−) at 37°C for 48 h as the control. These results are indicated as the mean ± standard error (n = 3). N.D.: not detected.

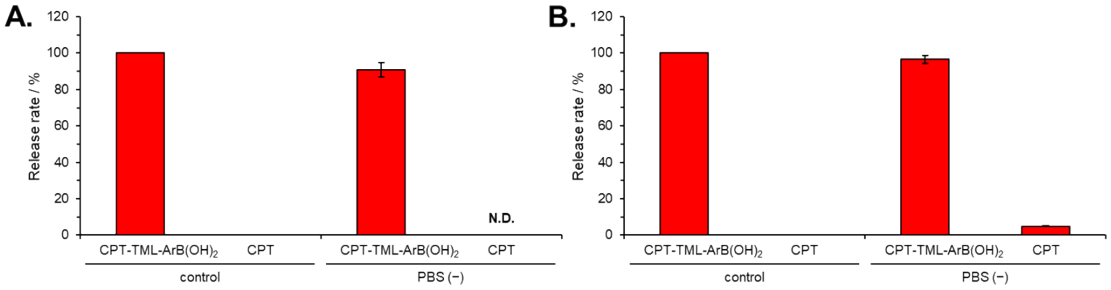


Fig. S4. Release rate of (A) **CPT-TML-ArB(OH)$_2$ NPDs** in water and (B) **CPT-TML-ArB(OH)$_2$** in DMSO incubated with PBS (−) at 37°C for 48 h. The relative amounts were calculated based on the concentration of the **CPT-TML-ArB(OH)$_2$** before incubation as the control. These results are indicated as the mean ± standard error (n = 3). N.D.: not detected.

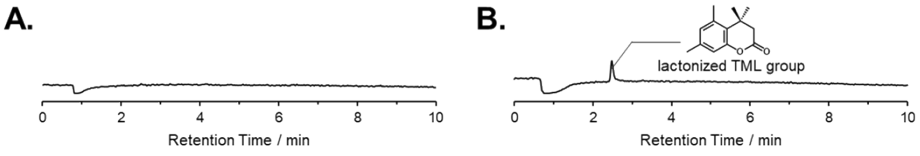


Fig. S5. HPLC-MS/MS chromatogram of lactonized **TML** group, (A) **CPT-TML-ArB(OH)$_2$** (untreated control) and (B) **CPT-TML-ArB(OH)$_2$** in DMSO incubated with 100 μM H_2O_2 solution at 37°C for 1 h. HPLC-MS/MS measurements were performed using methods which monitored only the peak of lactonized **TML** group.

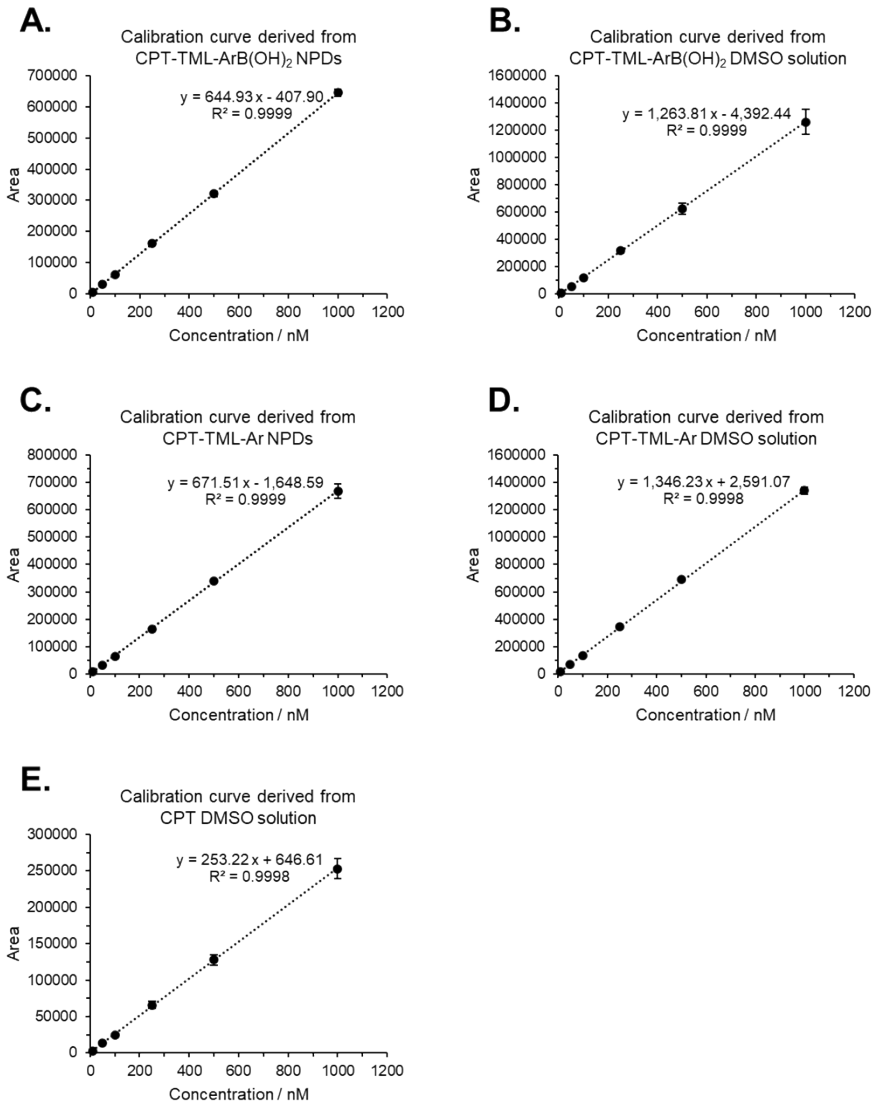


Fig. S6. Calibration curves for quantification of drug release kinetics in **CPT-TML**. Calibration curves were determined from calibration standard solutions by serially diluting each of (A) **CPT-TML-ArB(OH)$_2$ NPDs**, (B) **CPT-TML-ArB(OH)$_2$** DMSO solution, (C) **CPT-TML-Ar NPDs**, (D) **CPT-TML-Ar** DMSO solution and (E) **CPT** DMSO solution. These results are indicated as the mean ± standard error (*n* = 3).

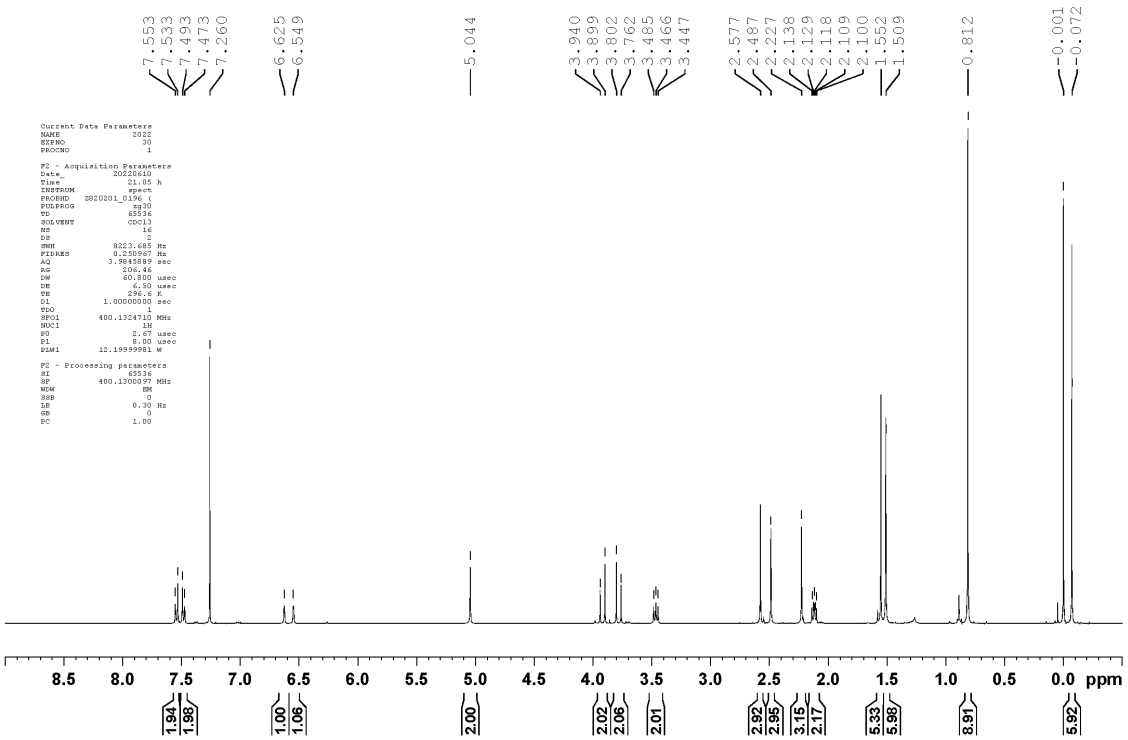


Fig. S7–A. 400 MHz ^{1}H NMR spectrum ($CDCl_3$) of compound (**6**).

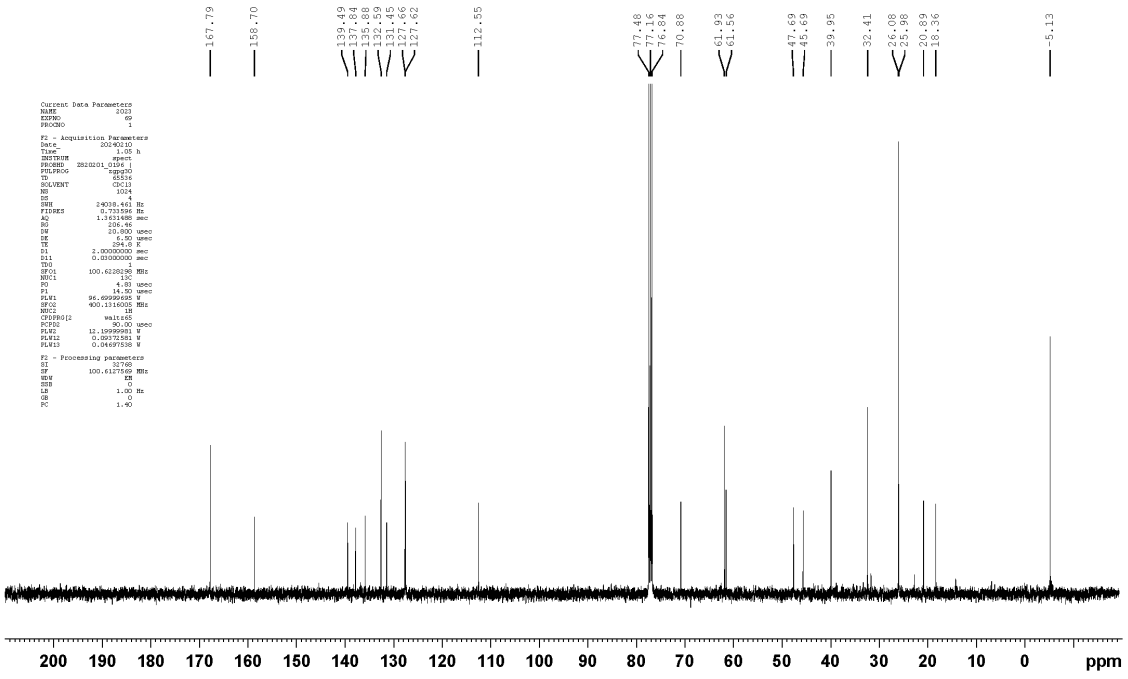


Fig. S7–B. 400 MHz ^{13}C NMR spectrum ($CDCl_3$) of compound (**6**).

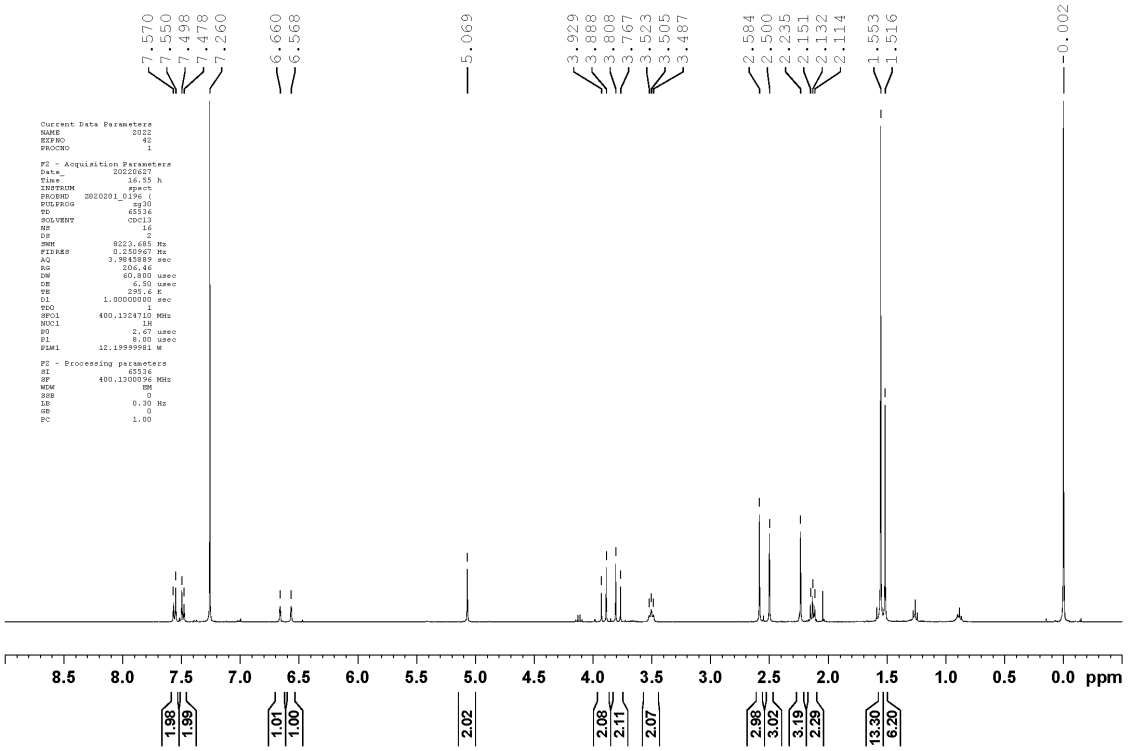


Fig. S8–A. 400 MHz ^{1}H NMR spectrum ($CDCl_3$) of compound (**7**).

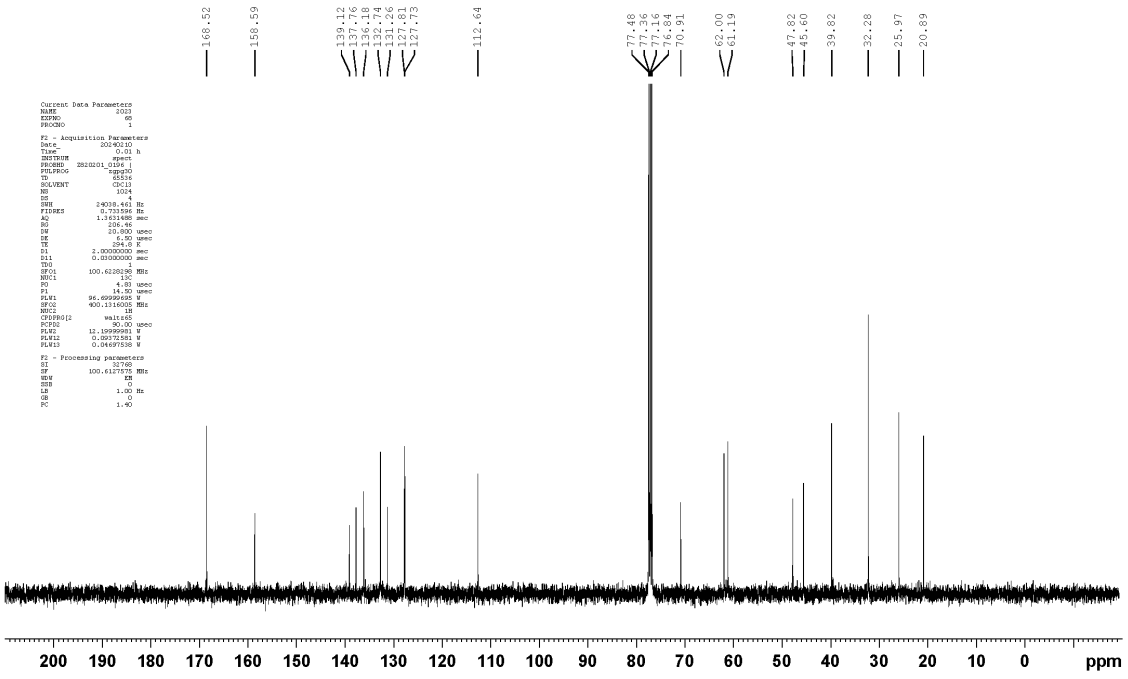


Fig. S8–B. 400 MHz ^{13}C NMR spectrum ($CDCl_3$) of compound (**7**).

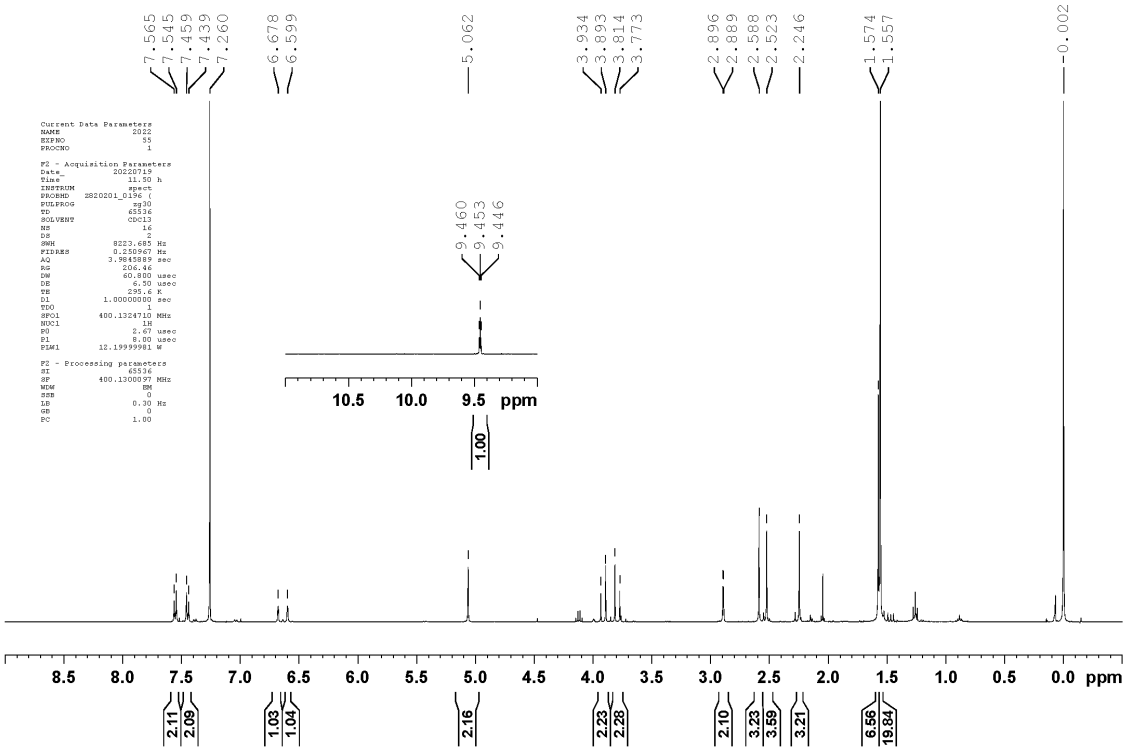


Fig. S9–A. 400 MHz ^{1}H NMR spectrum ($CDCl_3$) of compound (**8**).

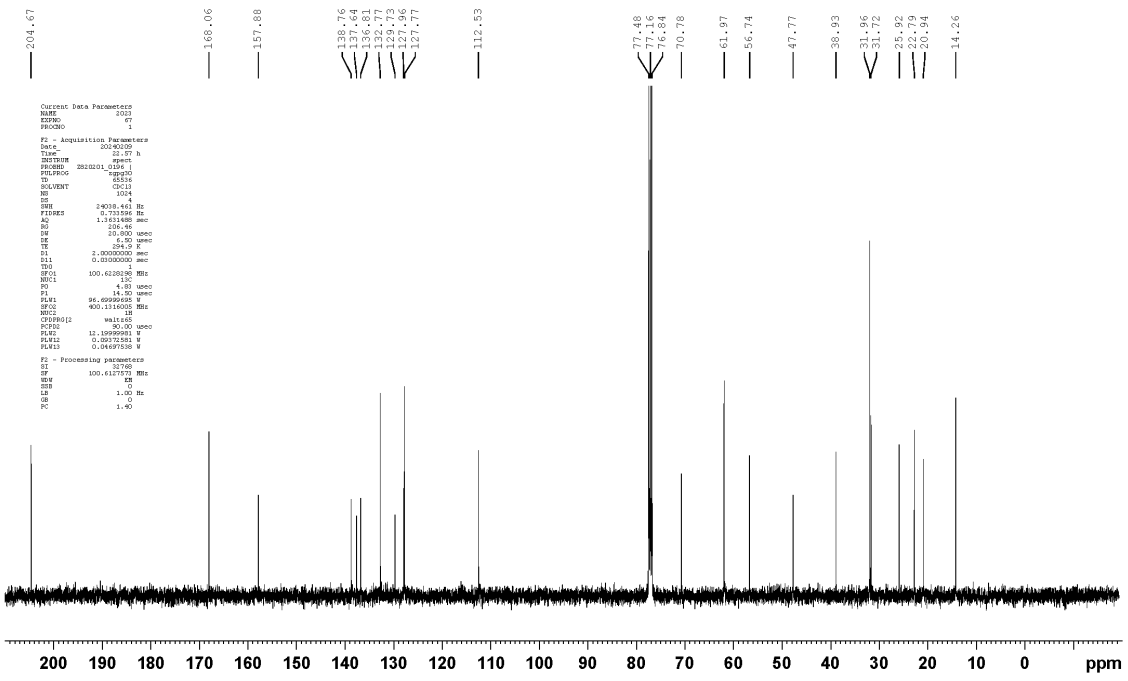


Fig. S9–B. 400 MHz ^{13}C NMR spectrum ($CDCl_3$) of compound (**8**).

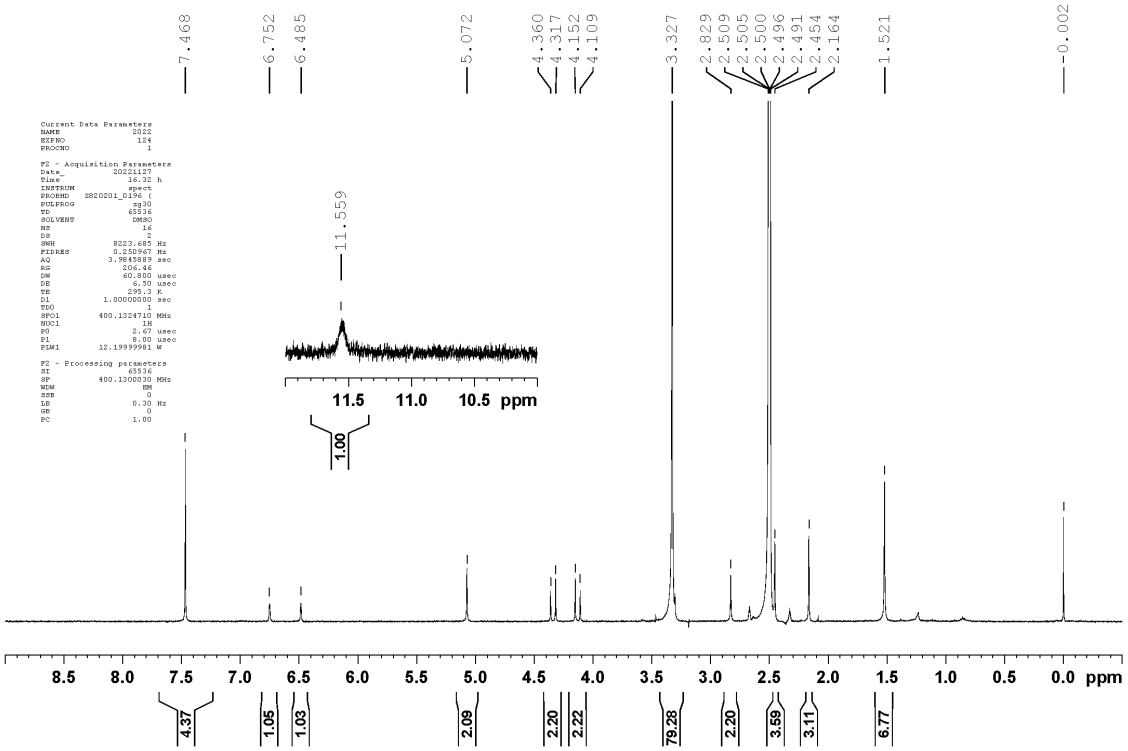


Fig. S10–A. 400 MHz ^{1}H NMR spectrum (DMSO-d_6) of compound (**9**).

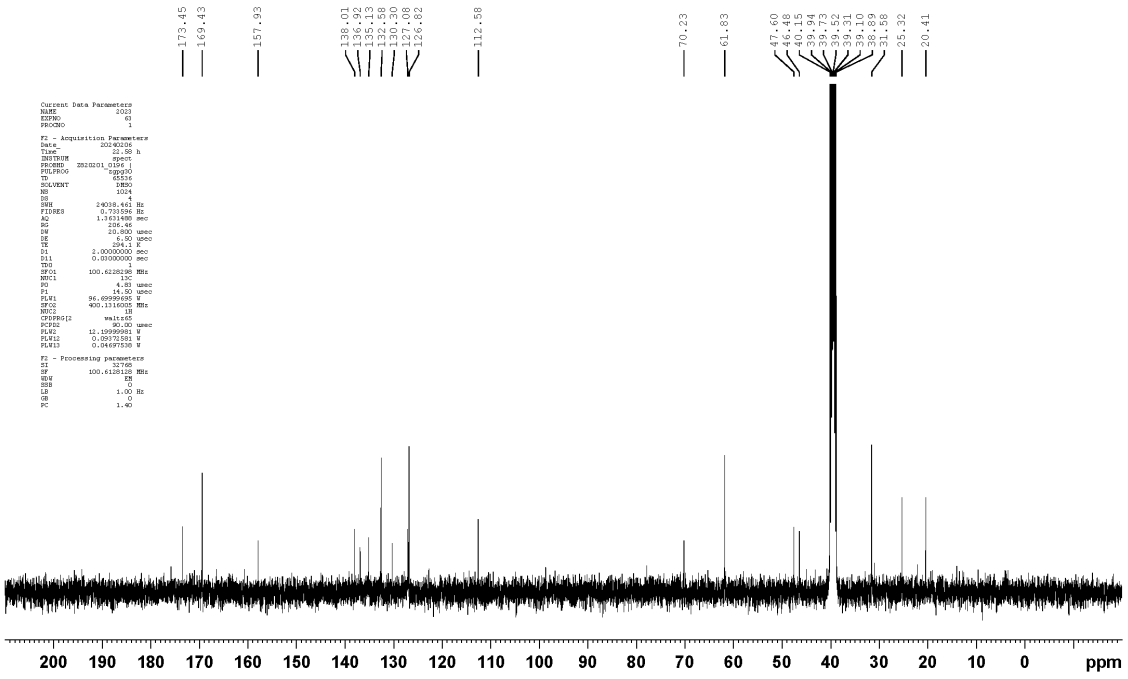


Fig. S10–B. 400 MHz ^{13}C NMR spectrum (DMSO-d_6) of compound (**9**).

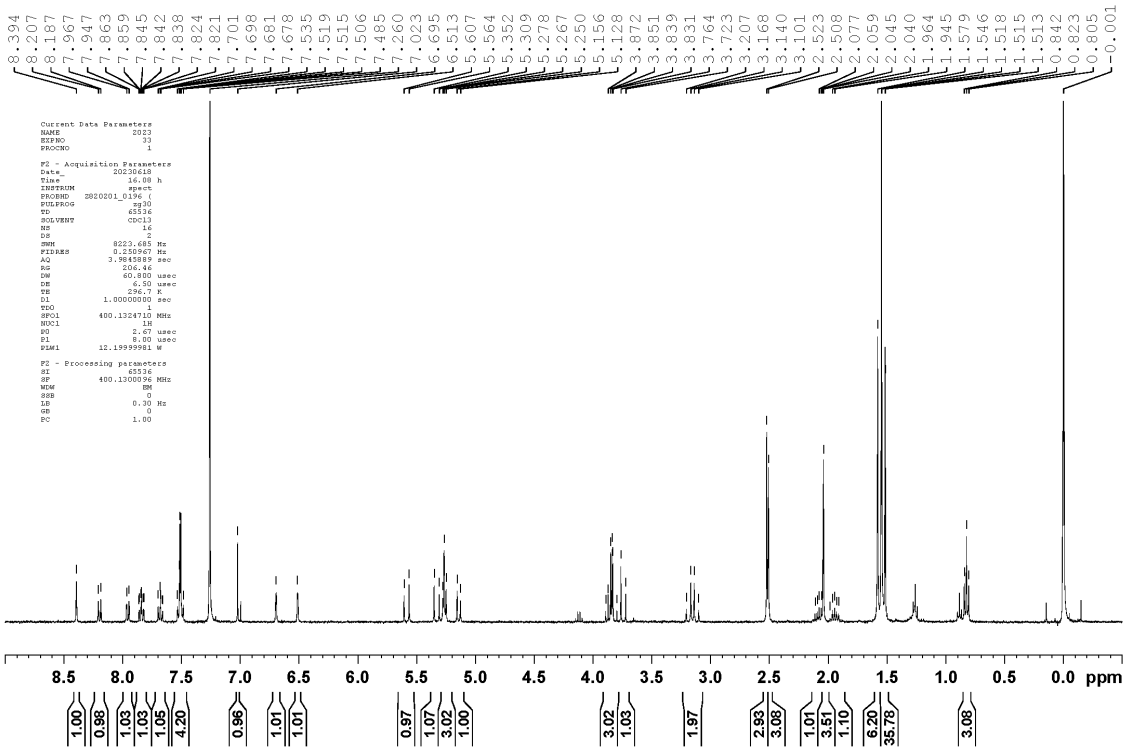


Fig. S11–A. 400 MHz ^{1}H NMR spectrum ($CDCl_3$) of compound (**10**).

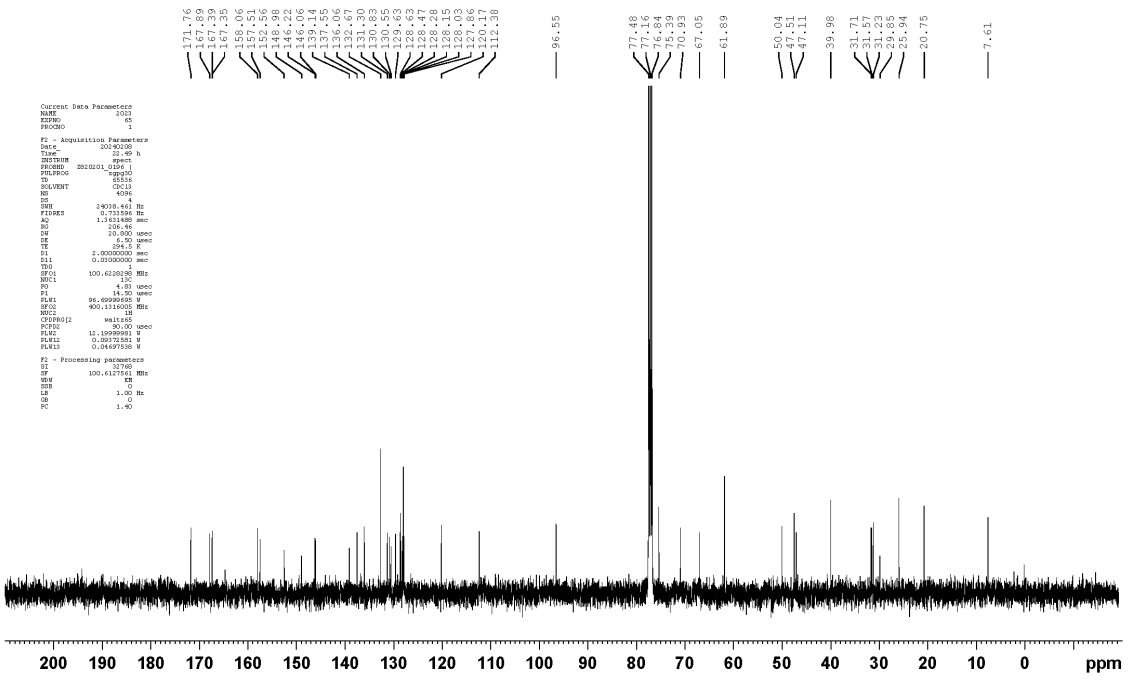


Fig. S11–B. 400 MHz ^{13}C NMR spectrum ($CDCl_3$) of compound (**10**).

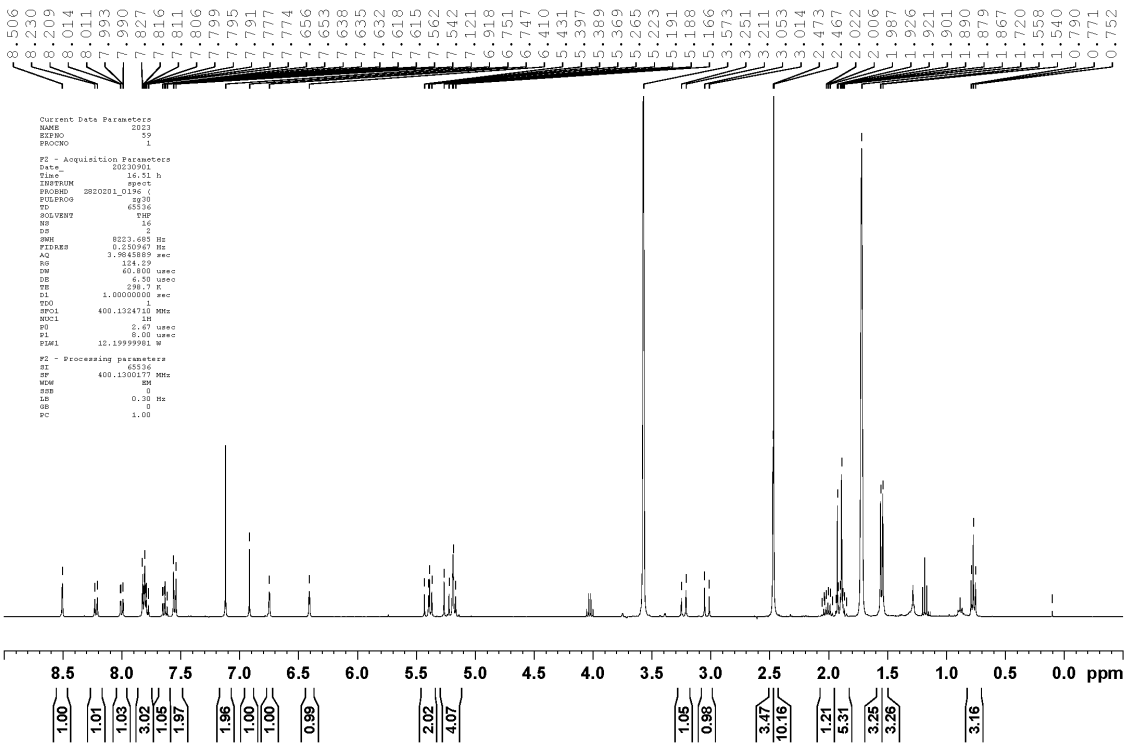


Fig. S12–A. 400 MHz ^{1}H NMR spectrum (THF-d_8) of compound (**11**).

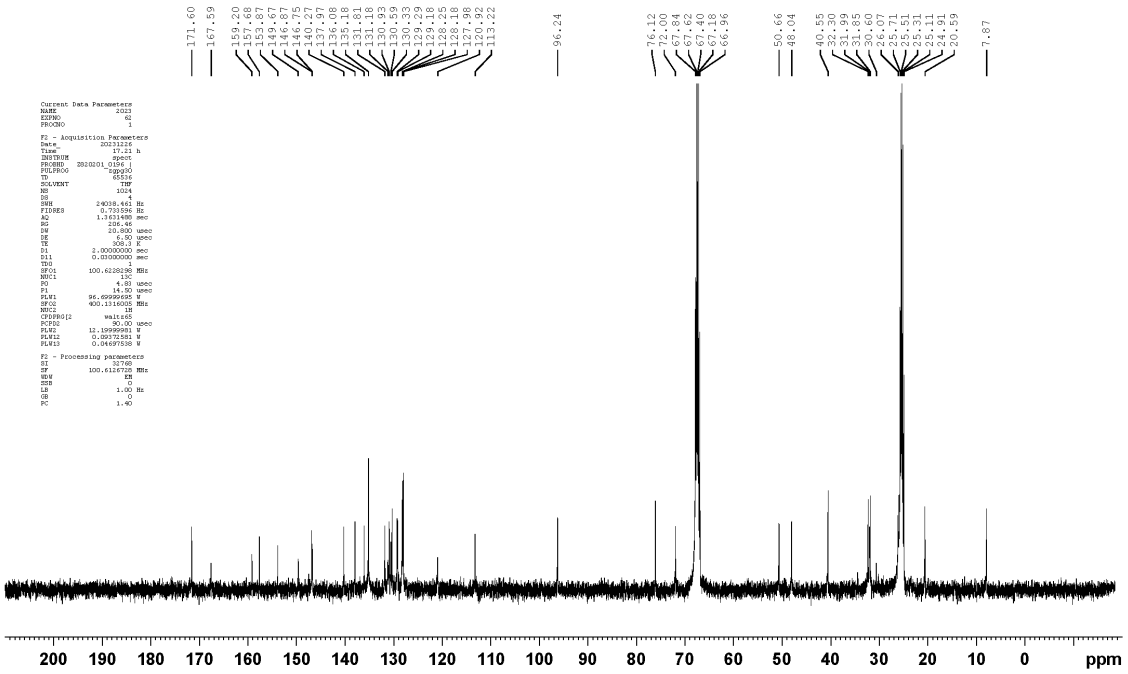


Fig. S12–B. 400 MHz ^{13}C NMR spectrum (THF-d_8) of compound (**11**).

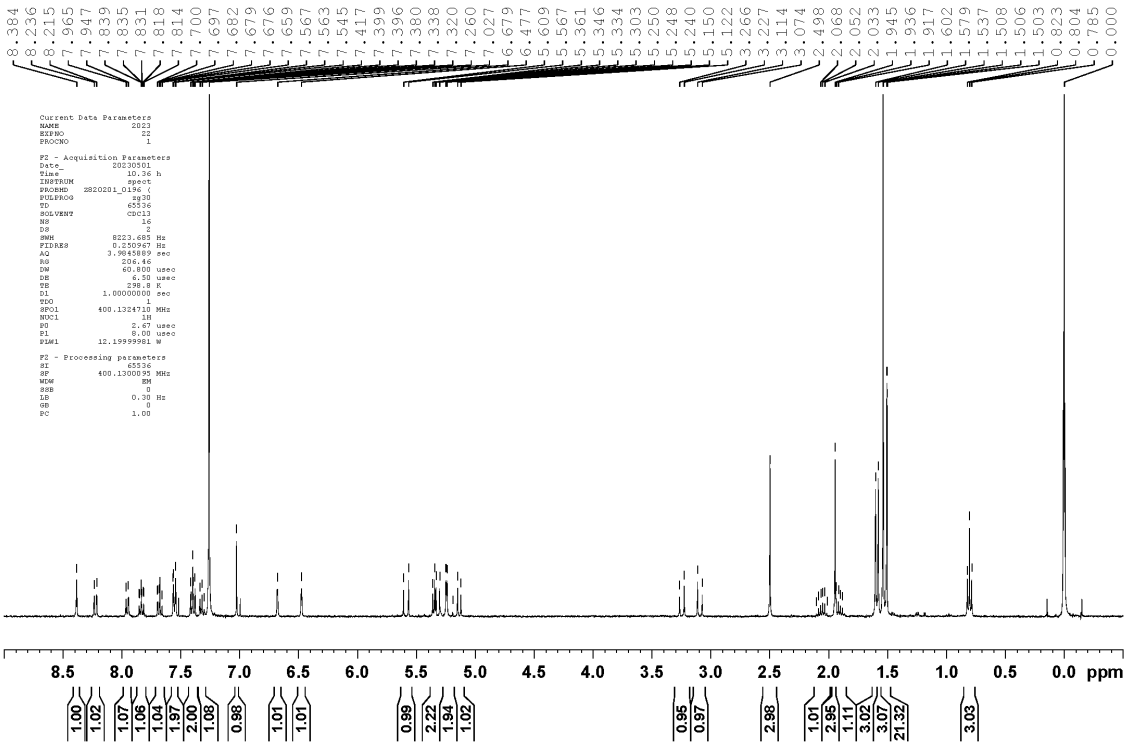


Fig. S13–A. 400 MHz ^{1}H NMR spectrum ($CDCl_3$) of compound (**13**).

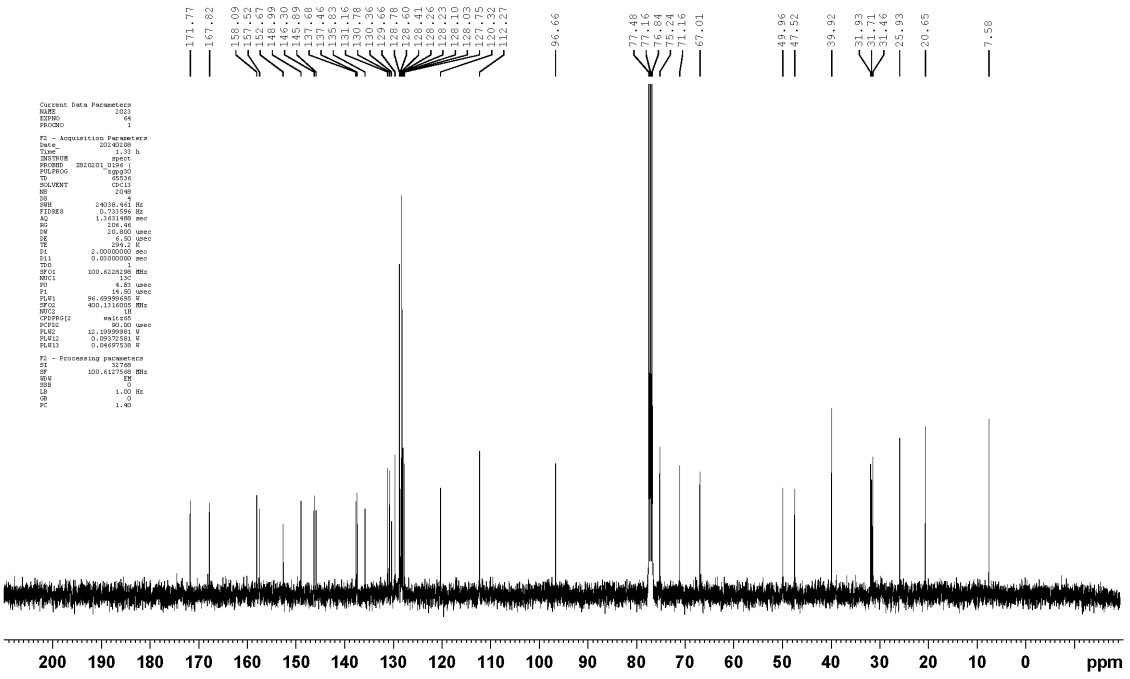


Fig. S13–B. 400 MHz ^{13}C NMR spectrum ($CDCl_3$) of compound (**13**).

SI Reference

1 R. Liu, J. Aw, W. Teo, P. Padmanabhan and B. Xing, *New Journal of Chemistry*, 2010, **34**, 594–598.

2 M. G. Nicolaou, C.-S. Yuan and R. T. Borchardt, *The Journal of Organic Chemistry*, 1996, **61**, 8636–8641.

3 M. Kita, J. Yamamoto, T. Morisaki, C. Komiya, T. Inokuma, L. Miyamoto, K. Tsuchiya, A. Shigenaga and A. Otaka, *Tetrahedron Letters*, 2015, **56**, 4228–4231.

4 E. P. Gillis and M. D. Burke, *Journal of the American Chemical Society*, 2008, **130**, 14084–14085.

5 K. L. Amsberry and R. T. Borchardt, *The Journal of Organic Chemistry*, 1990, **55**, 5867–5877.

6 H. Kasai, H. S. Nalwa, H. Oikawa, S. Okada, H. Matsuda, N. Minami, A. Kakuta, K. Ono, A. Mukoh and H. Nakanishi, *Japanese Journal of Applied Physics*, 1992, **31**, L1132.