ELECTRONIC SUPPLEMENTARY INFORMATION (ESI†)

Poly-ion complex micelle effectively delivers CoA-conjugated CPT1A inhibitors to modulate lipid metabolism in brain cells

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OUTLINE

1. Synthesis of C75

4-Benzyl-3-(trans-2-octyl-5-oxotetrahydrofuran-3-carbonyl)oxazolidin-2-one [**4** and **5**]. Compound (±)-**3** (1.000 g, 4 mmol) was added to a round-bottom flask. Anhydrous THF (45.6 mL) was added under N_2 atmosphere. The solution was cooled to -78 °C. Triethylamine (0.59 mL, 4.23 mmol) and pivaloyl chloride (0.52 mL, 4.22 mmol) were added. The solution was stirred for 1 h at 0 °C and then cooled down to -78 °C. In parallel, (*S*)-4-benzyl-2-oxazolidinone (700 mg, 4 mmol) was added to a round-bottom flask, anhydrous THF (9.4 mL) was added under N_2 atmosphere, and the solution was cooled to -78 °C. 2M BuLi in hexane (2.0 mL, 4 mmol) was slowly added to the (*S*)-4-benzyl-2-oxazolidinone solution. The (*S*)-4-benzyl-2-oxazolidinone solution was transferred to the compound (±)-**3** solution by using a cannula. The mixture was stirred for 15 minutes at -78 °C and then 30 minutes at 0 °C. The reaction was quenched with a saturated aqueous NH_4Cl solution (35 mL). The organic phase was washed with brine, and dried

over $MgSO_4$, filtered and evaporated under reduced pressure. After a column chromatography with silica gel using CH_2Cl_2 as eluent, compounds **4** (0.703 g, 1.75 mmol, 44%) and **5** (0.778 g, 1.93 mmol, 48%) were obtained.

Compound [**4**]: white solid; $[\alpha]_D$ = +82.3 (*c* 1.0, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.39 – 7.27 (m, 3H), 7.18 (m, 2H), 4.80 (m, 1H), 4.71 (m, 1H), 4.34 – 4.21 (m, 2H), 4.21 – 4.08 (m, 1H), 3.26 (dd, *J* = 13.4, 3.4 Hz, 1H), 3.00 (dd, *J* = 17.6, 9.4 Hz, 1H), 2.83 (dd, *J* = 13.4, 9.3 Hz, 1H), 2.71 (dd, *J* = 17.6, 7.0 Hz, 1H), 1.69 (m, 2H), 1.60 – 1.16 (m, 12H), 0.88 (t, *J* = 6.9 Hz, 3H). ^{13}C-NMR (101 MHz, $CDCl_3$): δ 174.3, 171.1, 153.0, 134.6, 129.3, 129.1, 127.6, 81.5, 66.7, 55.2, 45.0, 37.7, 35.1, 32.5, 31.8, 29.3, 29.2, 29.1, 25.3, 22.6, 14.1.

Compound [**5**]: white solid; $[\alpha]_D$ = +24.8 (*c* 1.0, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.47 – 7.09 (m, 5H), 4.71 (m, 2H), 4.37 – 4.23 (m, 2H), 4.24 – 4.14 (m, 1H), 3.26 (dd, *J* = 13.5, 3.3 Hz, 1H), 2.96 – 2.71 (m, 3H), 1.87 – 1.62 (m, 2H), 1.63 – 1.12 (m, 12H), 0.88 (t, *J* = 6.8 Hz, 3H). ^{13}C-NMR (101 MHz, $CDCl_3$): δ 174.5, 171.2, 153.1, 134.5, 129.4, 129.0, 127.6, 82.1, 66.7, 55.2, 44.9, 37.8, 35.1, 31.9, 31.8, 29.4, 29.2, 29.1, 25.3, 22.6, 14.1.

(+)-Trans-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid [(+)-**3**]. Compound **4** (1.918 g, 4.76 mmol) was added to a round-bottom flask with a THF/H_2O 1:1 solution (175 mL) and the solution was cooled to 0 °C. H_2O_2 30% w/w (4.3 mL, 41.8 mmol) and LiOH (0.238 g, 9.93 mmol) were added. The solution was stirred for 3 h at 0 °C and then 30 minutes at RT. Aqueous Na_2SO_3 15% w/w (25.2 mL) was added, and then the mixture was basified with NaOH 1N. Solvent was evaporated under reduced pressure. The aqueous solution was washed with CH_2Cl_2 (5×80 mL) and acidified to pH=1 with concentrated HCl. The solution was stirred overnight at RT. The aqueous solution was extracted with CH_2Cl_2 (4×80 mL). The combined organic layer was washed with brine, dried over $MgSO_4$, filtered and evaporated under reduced pressure. Compound (+)-**3** (1.070 g, 4.34 mmol, 91%) was obtained.

Compound [(+)-**3**]: white solid; $[\alpha]_D$ = +33.4 (*c* 1.0, MeOH); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.62 (td, *J* = 7.5, 4.8 Hz, 1H), 3.18 – 3.04 (m, 1H), 2.94 (dd, *J* = 17.8, 8.5 Hz, 1H), 2.82 (dd, *J* = 17.8, 9.6 Hz, 1H), 1.90 – 1.62 (m, 2H), 1.62 – 1.15 (m, 12H), 0.88 (t, *J* = 6.9 Hz, 3H).

(–)-Trans-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid [(–)-**3**]. Compound **5** (2.078 g, 5.17 mmol) was added to a round-bottom flask with a THF/H_2O 1:1 solution (250 mL) and the solution was cooled to 0 °C. H_2O_2 30% w/w (5.3 mL, 51.9 mmol) and LiOH (0.28 g, 11.69 mmol) were added. The solution was stirred for 3 h at 0 °C and then 30 minutes at RT. Aqueous Na_2SO_3 15%

w/w (31 mL) was added, and then the mixture was basified with NaOH 1N. Solvent was evaporated under reduced pressure. The aqueous solution was washed with CH_2Cl_2 (5×100 mL) and acidified to pH=1 with concentrated HCl. The solution was stirred overnight at RT. The aqueous solution was extracted with CH_2Cl_2 (4×100 mL). The combined organic layer was washed with brine, dried over $MgSO_4$, filtered and evaporated under reduced pressure. Compound (–)-**3** (1.22 g, 4.95 mmol, 95%) was obtained.

Compound [(–)-**3**]: white solid; $[\alpha]_D$ = –40.0 (*c* 1.0, MeOH); ^{1}H-NMR (400 MHz, $CDCl_3$): δ 4.62 (td, *J* = 7.5, 4.7 Hz, 1H), 3.16 – 3.04 (m, 1H), 2.94 (dd, *J* = 17.9, 8.5 Hz, 1H), 2.82 (dd, *J* = 17.9, 9.6 Hz, 1H), 1.90 – 1.62 (m, 2H), 1.58 – 1.15 (m, 12H), 0.88 (t, *J* = 6.9 Hz, 2H).

(2R,3S)-4-Methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid [(+)-C75]. Compound (+)-**3** (0.300 g, 1.21 mmol) was added to a round-bottom flask. Under N_2 atmosphere, 2.0 M MMC in DMF (20 mL, 0.04 mmol) was added. The mixture was stirred for 48 h at 135 °C. The mixture was cooled to RT. HCl 6M (30 ml) cooled to 0 °C was added slowly while stirring. A dark solid was formed, and it dissolved slowly upon addition of the HCl 6M solution. CH_2Cl_2 (30 mL) was added, and the aqueous phase was extracted with CH_2Cl_2 (2×15 mL). The combined organic layer was dried over $MgSO_4$, filtered and evaporated under reduced pressure at RT. A fresh solution of acetic acid (3 mL), formol (2.25 mL), *N*-methylaniline (0.78 mL) and AcONa (90 mg) was prepared, and this solution (4.4 mL) was added to the reaction mixture. The mixture was stirred for 1 h 45 minutes at RT. A 10:1 solution of brine/concentrated HCl (15 mL) was added. CH_2Cl_2 (20 mL) was added, and the mixture was extracted with CH_2Cl_2 (3×10 mL). The combined organic layer was washed with LiCl 5% (2×12.5 mL), 0.02N HCl (2×12.5 mL), and H_2O (3×15 mL). The organic phase was then stirred for 5 min at RT with a saturated solution of $NaHCO_3$ (20 mL). The aqueous phase was acidified to pH=1 with concentrated HCl. The aqueous solution was extracted with CH_2Cl_2 (4×15 mL). The combined organic layer was dried over $MgSO_4$, filtered and evaporated under reduced pressure. (+)-C75 (0.237 g, 0.93 mmol, 76%) was obtained.

Compound [(+)-**C75**]: white solid $[\alpha]_D$ = +11.4 (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.46 (d, *J* = 3.0 Hz, 1H), 6.02 (d, *J* = 2.7 Hz, 1H), 4.81 (td, *J* = 7.2, 5.6 Hz, 1H), 3.63 (dt, *J* = 5.6, 2.8 Hz, 1H), 1.79 – 1.67 (m, 2H) , 1.53 – 1.20 (m, 12H), 0.88 (t, *J* = 6.9 Hz, 3H).

(2S,3R)-4-Methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid [(–)-C75]. Compound (–)-**3** (0.600 g, 2.43 mmol) was added to a round-bottom flask. Under N_2 atmosphere, 2.0 M MMC in DMF (40 mL, 0.08 mmol) was added. The mixture was stirred for 48 h at 135 °C. The mixture

was cooled to RT. HCl 6M 60 mL) cooled to 0 °C was added slowly while stirring. A dark solid was formed, and it dissolved slowly upon addition of the HCl 6M solution. CH_2Cl_2 (50 mL) was added, and the aqueous phase was extracted with CH_2Cl_2 (2×30 mL). The combined organic layer was dried over $MgSO_4$, filtered and evaporated under reduced pressure at RT. A fresh solution of acetic acid (6.25 mL), formol (4.68 mL), *N*-methylaniline (1.63 mL) and AcONa (187.5 mg) was prepared, and this solution (8.75 mL) was added to the reaction mixture. The mixture was stirred for 1 h 45 minutes at RT. A 10:1 solution of brine/concentrated HCl (30 mL) was added. CH_2Cl_2 (37.5 mL) was added, and the mixture was extracted with CH_2Cl_2 (3×20 mL). The combined organic layer was washed with LiCl 5% (2×25 mL), 0.02N HCl (2×25 mL), and H_2O (3×30 mL). The organic phase was then stirred for 5 min at RT with a saturated solution of $NaHCO_3$ (35 mL). The aqueous phase was acidified to pH=1 with concentrated HCl. The aqueous solution was extracted with CH_2Cl_2 (4×20 mL). The combined organic layer was dried over $MgSO_4$, filtered and evaporated under reduced pressure. (±)-C75 (0.455 g, 1.78 mmol, 73%) was obtained.

Compound [(–)-**C75**]: white solid; $[\alpha]_D$ = –11.4 (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.46 (d, *J* = 3.0 Hz, 1H), 6.02 (d, *J* = 2.7 Hz, 1H), 4.81 (dt, *J* = 7.2, 5.6 Hz, 1H), 3.63 (dt, *J* = 5.6, 2.8 Hz, 1H), 1.79 – 1.67 (m, 2H), 1.53 – 1.20 (m, 12H), 0.88 (t, *J* = 6.9 Hz, 3H).

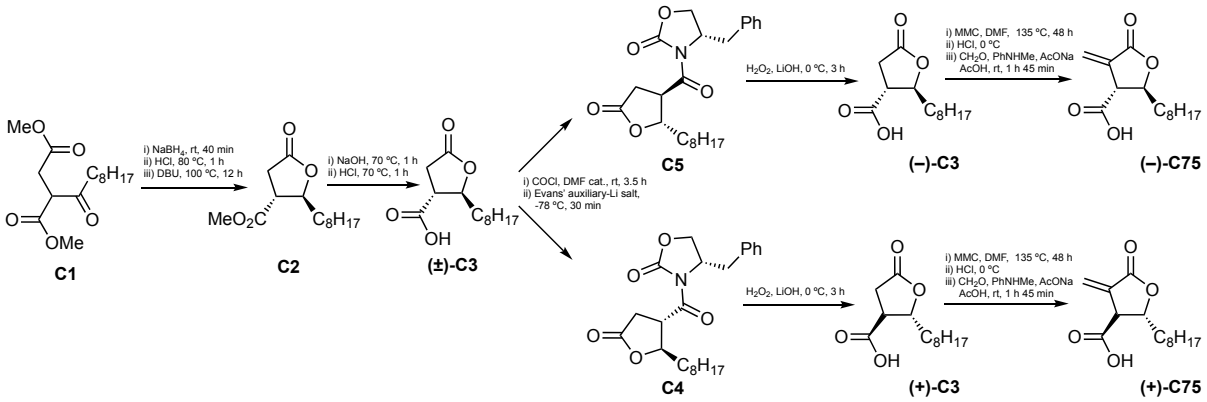


Figure S1. The enantioselective synthesis of the two enantiomers of C75.

Synthesis of (±)-C75-CoA: For ^{1}H NMR confirmation: Coenzyme A (HSCoA) sodium salt hydrate (8.6mg), and $Na_3PO_4 \cdot 12H_2O$ (7.6mg) were added to a solution of (±)-C75 (2.5mg) in D_2O (0.8ml)

in an NMR tube as previously reported [1]. ^{1}H NMR was recorded on a JEOL ECS 400 (400 MHz) spectrometer (JOEL Ltd., Tokyo Japan) and chemical shift was calculated as parts per million (ppm). Data was processed using MestReNova version 14.2.1-27684. For micelle preparation and biological assays, the reaction was carried out in 90 mM Na_3PO_4 and left at RT overnight. The same procedure was performed to synthesize (+)-C75-CoA and (–)-C75-CoA. HPLC analysis (LC-2000 series, JASCO, Tokyo, Japan) was performed to confirm whether all (±)-C75 was consumed in the reaction. Conditions include: C-18 RP-column (TSKgel ODS-100V 5µm particle size, 4.6 mm I.D. × 15 cm, TOSOH Bioscience, cat. # 21455), mobile phase 7:3 100 mM phosphate buffer pH 3/acetonitrile, flow rate 1.2 mL/minute, 259-nm detection.

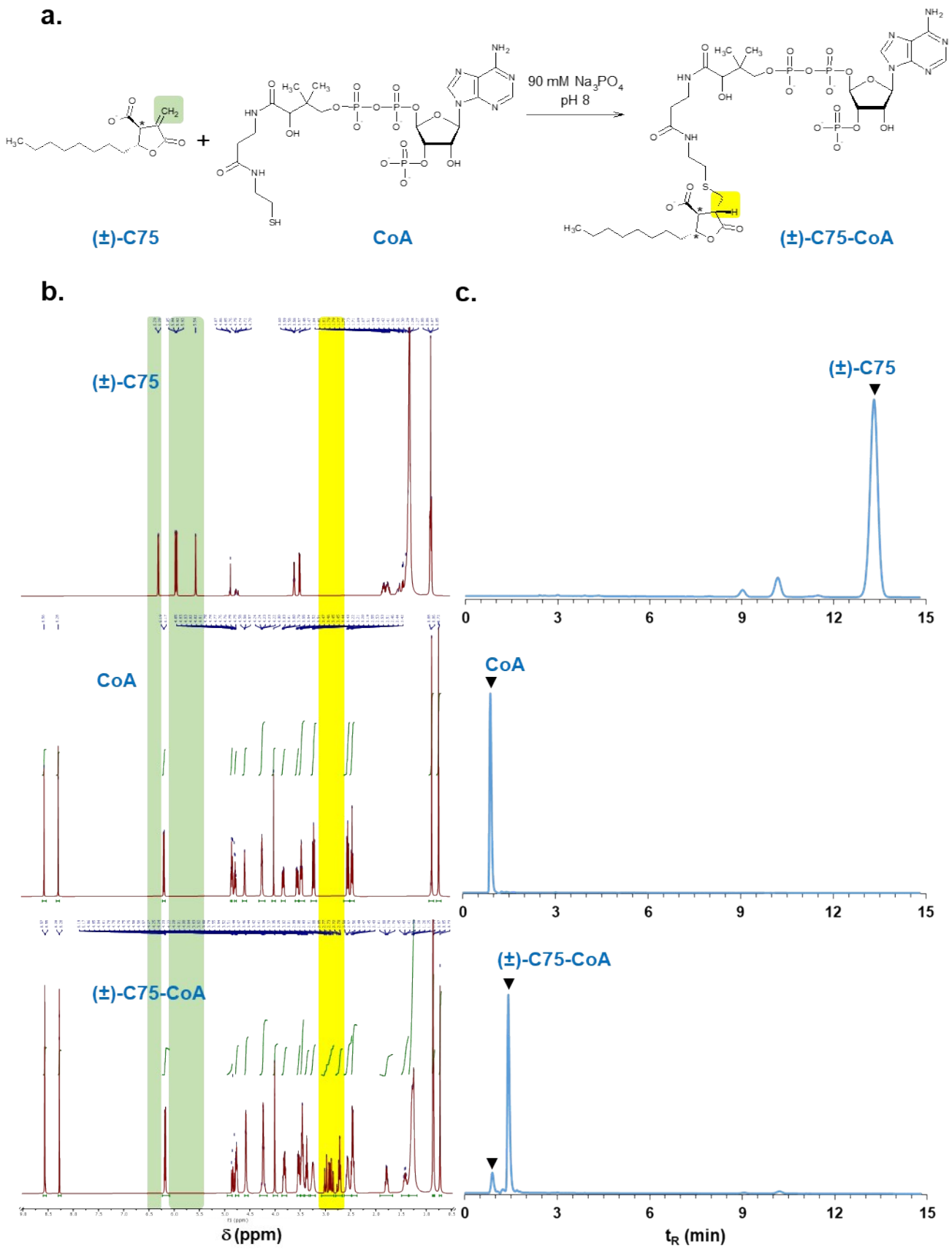

a.
90 mM Na3PO4
pH 8
(±)-C75
CoA
(±)-C75-CoA
b.
(±)-C75
CoA
(±)-C75-CoA
δ (ppm)
c.
(±)-C75
CoA
(±)-C75-CoA
0
3
6
9
12
15
tR (min)

Figure S2. Synthesis of (±)-C75-CoA (a), which involves the nucleophilic addition to the α,β-unsaturation of (±)-C75 by the thiol group of coenzyme A (CoA), to form (±)-C75-CoA. In the ^{1}H-NMR measurements (b), geminal alkene protons in (±)-C75 were highlighted in green while newly formed C-H bonds in (±)-C75-CoA were highlighted in yellow. HPLC profiles (c) of the starting materials were compared with that of the product.

2. Synthesis of PEG-PAsp(DET)

The diblock co-polymer was prepared by aminolysis of CH_3O-PEG-*b*-poly(β-benzyl-L-aspartate) (PEG-PBLA). PEG-PBLA was synthesized by anionic ring-opening polymerization of BLA-NCA initiated from the terminal ─NH_2 group of CH_3O-PEG-NH_2 [2]. CH_3O-PEG-NH_2 (MW 12,000 Da) and BLA-NCA were dissolved in distilled DCM-DMF (10:1), mixed, and allowed to react in Ar atmosphere for 72 h. After which, the polymer is precipitated, washed in 2:3 ethyl acetate-hexane three times, and collected by vacuum filtration. Complete drying was performed *in vacuo*.

Aminolysis of PEG-PBLA was carried out as previously reported [3,4]. Briefly, 150 mg freeze-dried PEG-PBLA was dissolved in 15 mL distilled NMP and cooled to 0 °C. In another reaction tube, distilled DET (4.7 mL, 100 times the molar equivalence of benzyl ester units) was mixed with NMP and cooled to 0 °C. PEG-PBLA in NMP solution was then added dropwise over 1 minute to the DET solution. The reaction was allowed to proceed in ice over 1 h reaction. After which, the polymer was added dropwise to 5 N aqueous HCl (34.1 mL, 1.3 × equivalent to the added 1° and 2° amine groups of DET) at <5 °C. The resulting acidified mixture was afterwards dialyzed (MWCO: 6,000−8,000) at 4 °C against a 0.01 N HCl 3-4 × and then against deionized water 2 ×. The final solution was freeze-dried to obtain PEG-PAsp(DET). Gel permeation chromatography (LC-2000 series, JASCO, Tokyo, Japan) was carried out using a Superdex™ 200 Increase 10/300GL (Cytiva, 28-9909-44, column L × I.D. 30 cm × 10 mm, 8.6 μm particle size) 10 mM CH_3COOH in 500 mM NaCl, 0.5 mL/min. ^{1}H NMR was recorded on a JEOL ECS 400 (400 MHz) spectrometer (JOEL Ltd., Tokyo Japan) and chemical shift was calculated as parts per million (ppm). Data was processed using MestReNova version 14.2.1-27684.

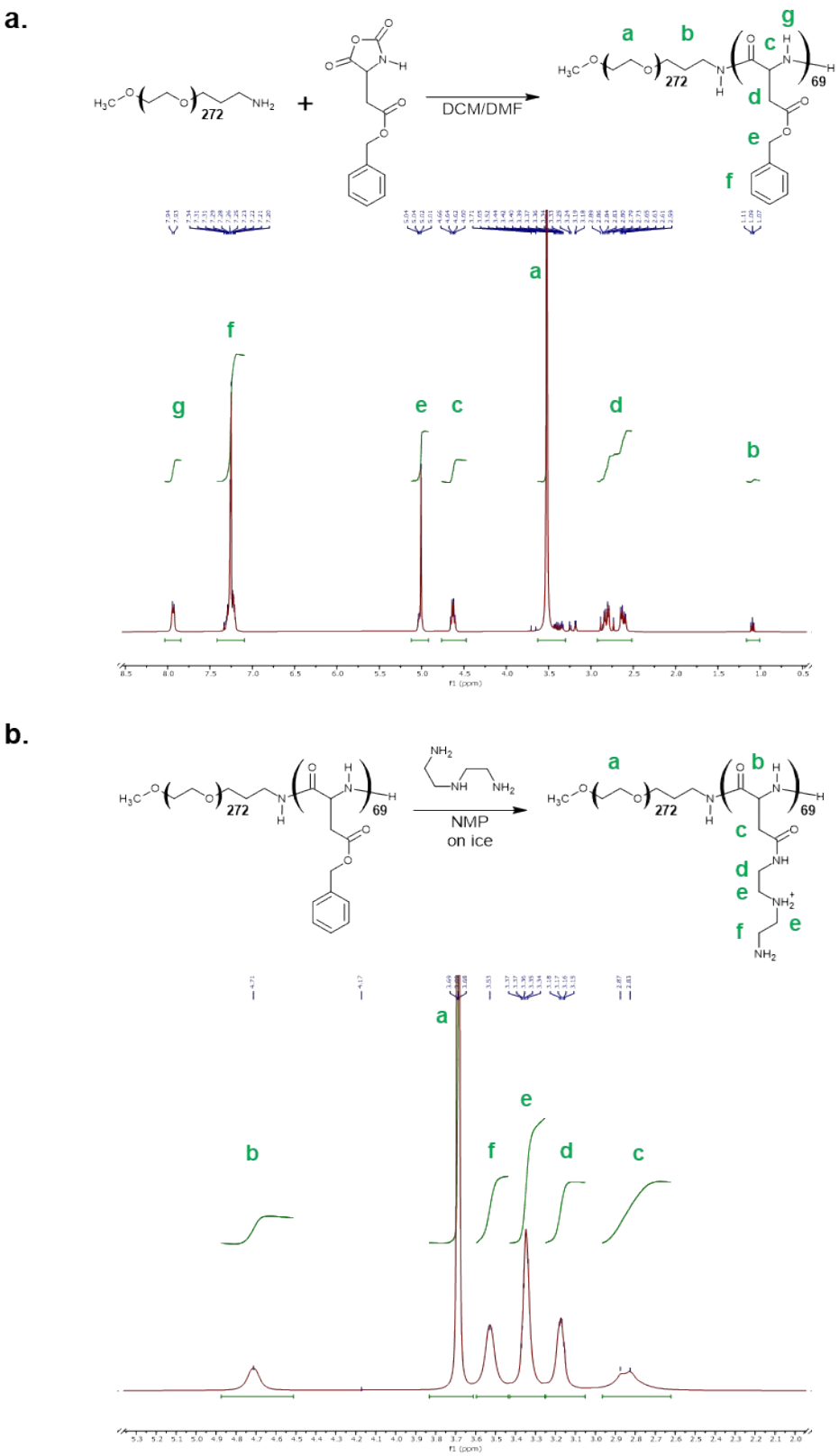
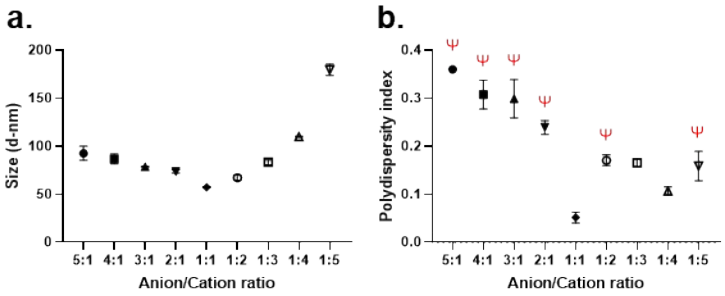


Figure S3. Synthesis of PEG-PBLA (a) and PEG-PAsp(DET) (b) and the corresponding ^{1}H-NMR measurements (PEG-PBLA in DMSO-d_6, 80 °C and PEG-Asp(DET) in D_2O, 80 °C).



c.

A/C Ratio	% weight	
	Polymer	Drug
5 : 1	19.80%	80.20%
4 : 1	23.58%	76.42%
3 : 1	29.15%	70.85%
2 : 1	38.17%	61.83%
1 : 1	55.25%	44.75%
1 : 2	71.17%	28.83%
1 : 3	78.74%	21.26%
1 : 4	83.16%	16.84%
1 : 5	86.06%	13.94%

Figure S4. Physicochemical properties of (±)-C75-CoA micelles prepared in different anion/cation (A/C) ratios including size (a) and polydispersity (b). Experiments were performed in triplicate (values expressed in mean ± SD). Polydisperse size profiles are marked with Ψ. Table showing % by weight of micelle components at different A/C ratios (c).

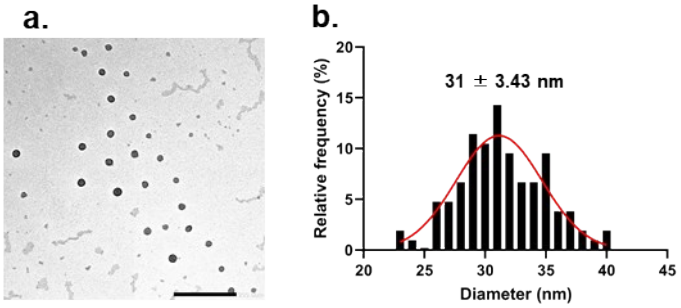


Figure S5. Transmission electron microscopy (TEM) image of (±)-C75-CoA micelle (a) and its size distribution profile (value expressed in mean ± SD, n = 105) (b). Scale bar = 100 µm, magnification 40×.

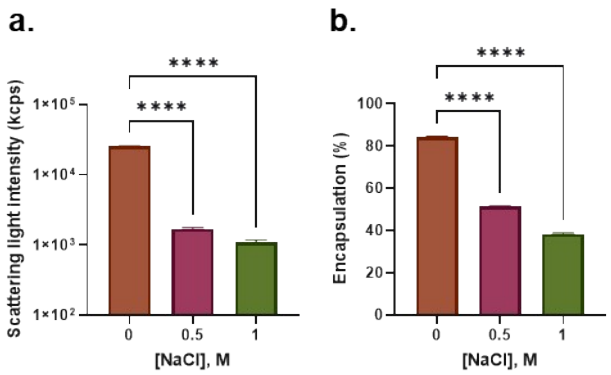


Figure S6. Changes in scattering light intensity (SLI) (a) and encapsulation (%) (b) of (±)-C75-CoA micelle (a) upon mixing with high NaCl concentration solutions. Experiments were performed in triplicate (values expressed in mean ± SD) and comparison of means among treatment groups were done using ANOVA (with Tukey's test as post-hoc analysis; **** p<0.0001).

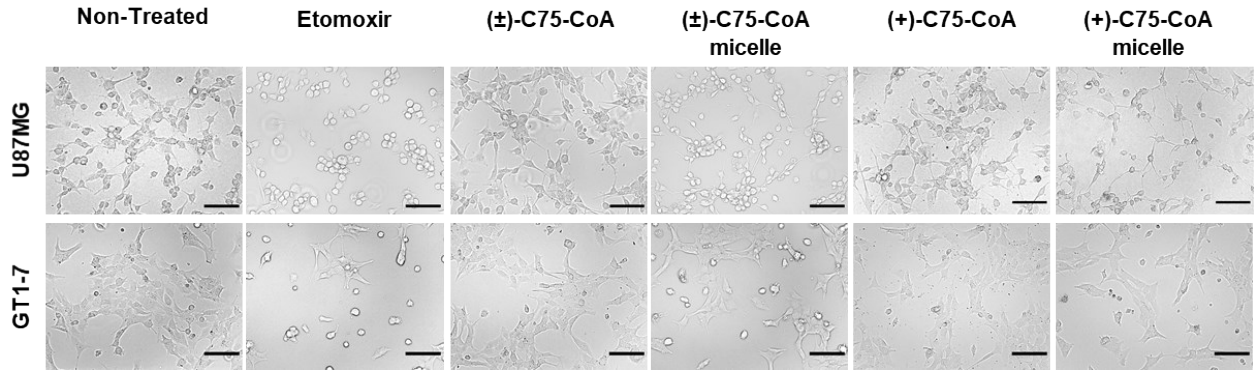


Figure S7. Microscopic images of U87MG and GT1-7 cells after treatment with FAO inhibitors. Scale bar = 100 µm, magnification 40×.

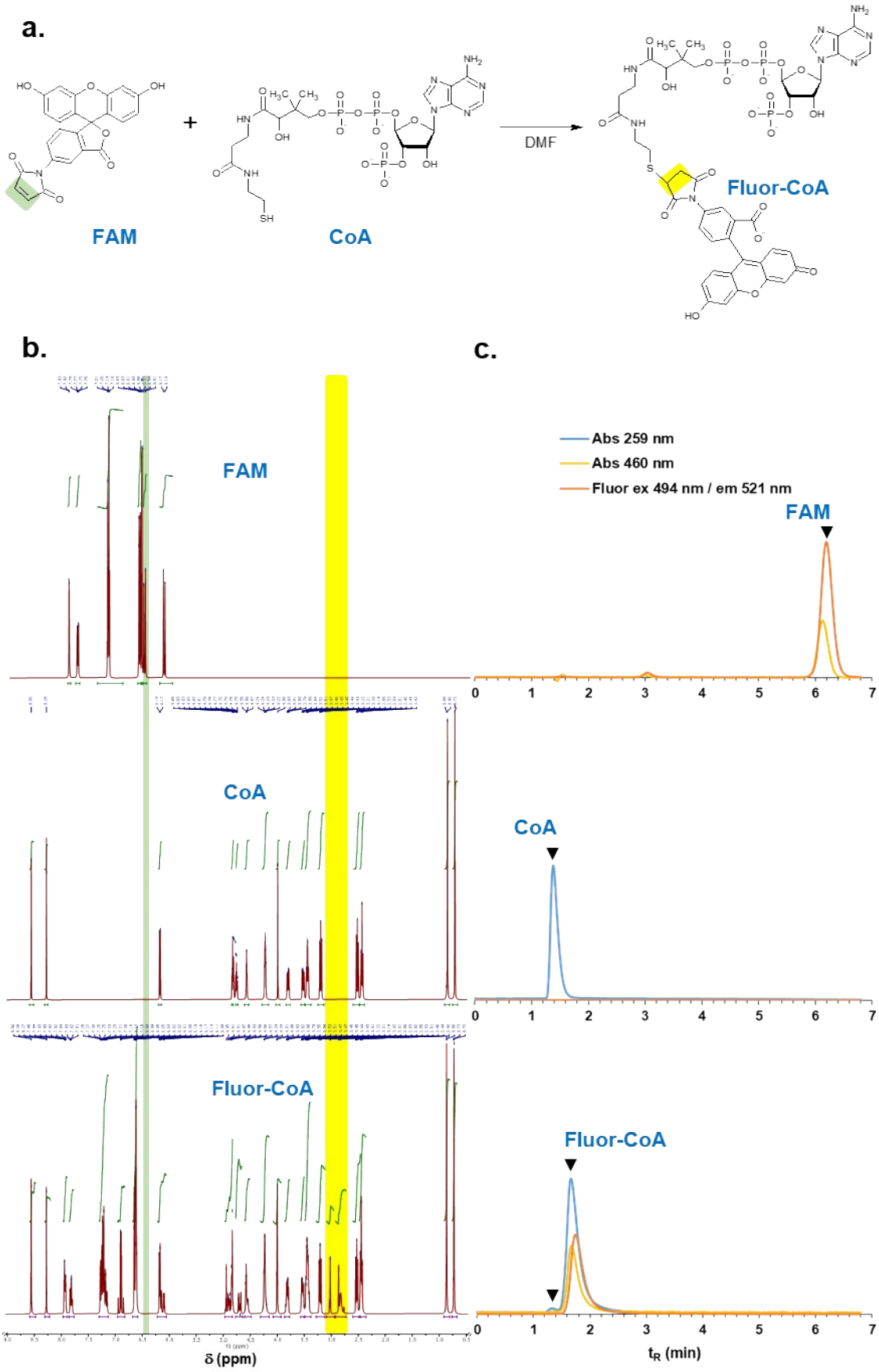

a.
FAM
CoA
DMF
Fluor-CoA
b.
FAM
CoA
Fluor-CoA
δ (ppm)
c.
Abs 259 nm
Abs 460 nm
Fluor ex 494 nm / em 521 nm
FAM
CoA
Fluor-CoA
0 1 2 3 4 5 6 7
tR (min)

Figure S8. Synthesis of Fluorescein-CoA (Fluor-CoA) (a), which is the nucleophilic addition to the maleimide ring of FAM by the thiol group of coenzyme A (CoA), to form Fluor-CoA. The ^{1}H-NMR measurements (b). Maleimide ring protons in FAM were highlighted in green while newly formed C-H bonds in Fluor-CoA were highlighted in yellow. HPLC profiles (c) of the starting materials were compared with that of the product.

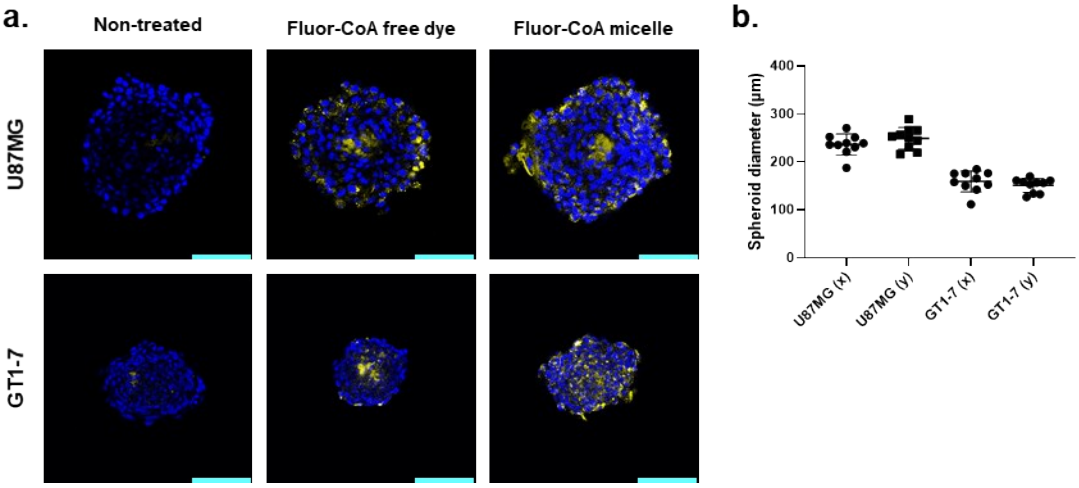


Figure S9. Microscopic images and analysis of cellular uptake of Fluor-CoA micelles in U87MG and GT1-7 spheroids. Representative U87MG and GT1-7 spheroid microscopic images (a) with scale bar = 100 µm, magnification 1.2×, nucleus (blue), Fluor-CoA (yellow) fluorescence signals are shown. Spheroid diameters in x and y dimensions (n = 10) were measured using Zen Zeiss software.

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