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Supporting Information for

Fluorescence Detection of Metabolic Activity of Fatty Acid Beta Oxidation Pathway in Living Cells

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Experimental Details

Synthesis and Characterization of the Compounds

Table S1. Photophysical property of probe **10** and coumarin **16**[a].

	molar extinction coefficient at 405 nm ($\varepsilon_{405\ nm}$, $M^{-1} \cdot cm^{-1}$)	quantum yield (Φ)	brightness ($\varepsilon_{405\ nm}$ x Φ)
probe **10**	31,500	0.49	1.5×10^4
coumarin **16**	27	0.47	13

[a]Meauremet was conducnted in the mixed solvent system (50 mM HEPES buffer (pH = 7.2) : methanol = 1 : 1) and 50 mM HEPES buffer (pH = 7.2) for probe **10** and **16**, respectively.

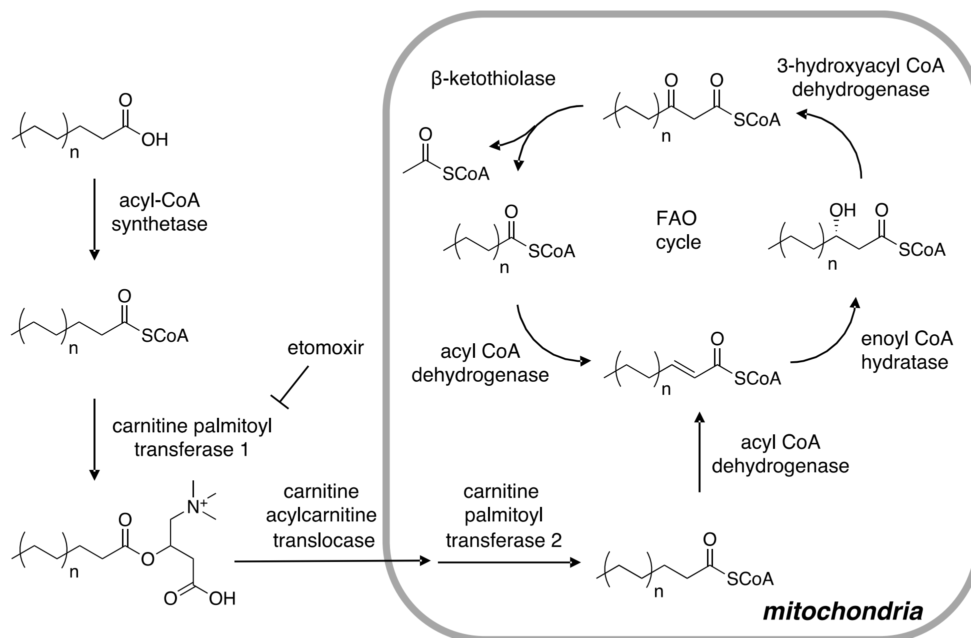


Figure S1. Metabolic pathway of fatty acid β-oxidation (FAO).

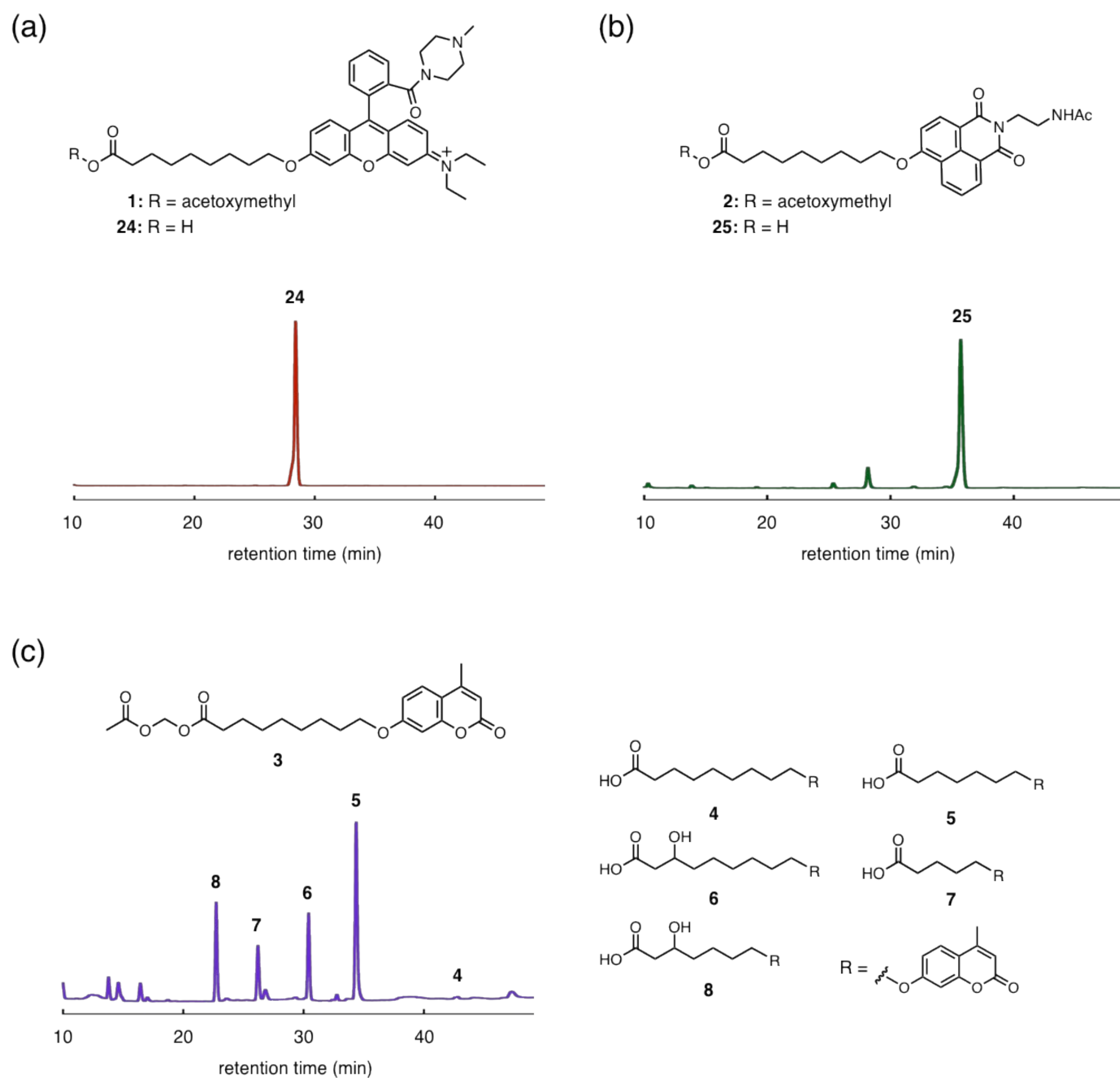


Figure S2. Evaluation of FAO degradation of probe **1** (a), **2** (b) and **3** (c) in HepG2 cells by HPLC analysis. The cells were incubated with probe **1-3** (20 µM) in serum-free DMEM for 6 h at 37 °C. The peaks were detected by UV absorbance at 520, 370, and 320 nm for probe **1**, **2** and **3**, respectively. Compound **4–8** were identified by ESI-TOF-MS analysis.

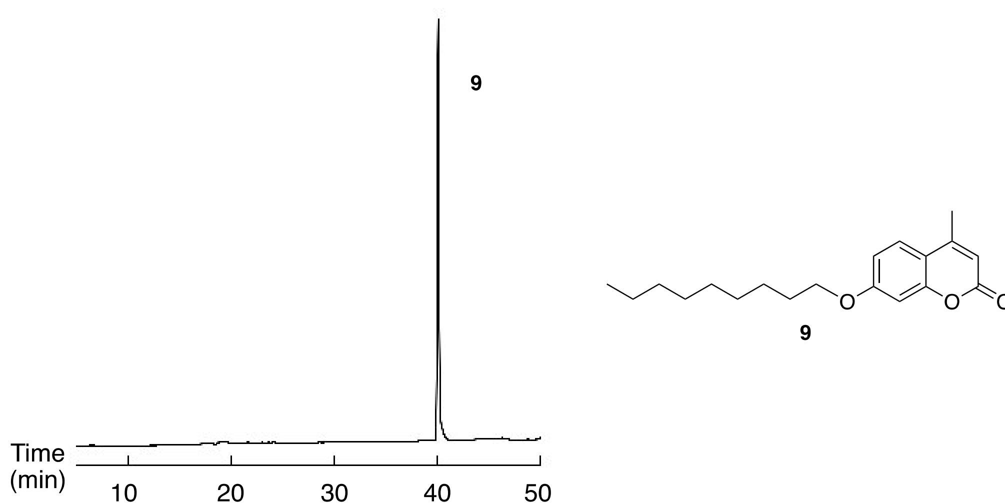


Figure S3. HPLC analysis of FAO metabolism of probe **9** in HepG2 cells. The cells were incubated with **9** (20 μM) in serum-free and phenol red-free DMEM for 6h at 37 °C. The main peak detected by UV absorbance (λ = 320 nm) was identified as **9** by ESI-TOF-MS analysis.

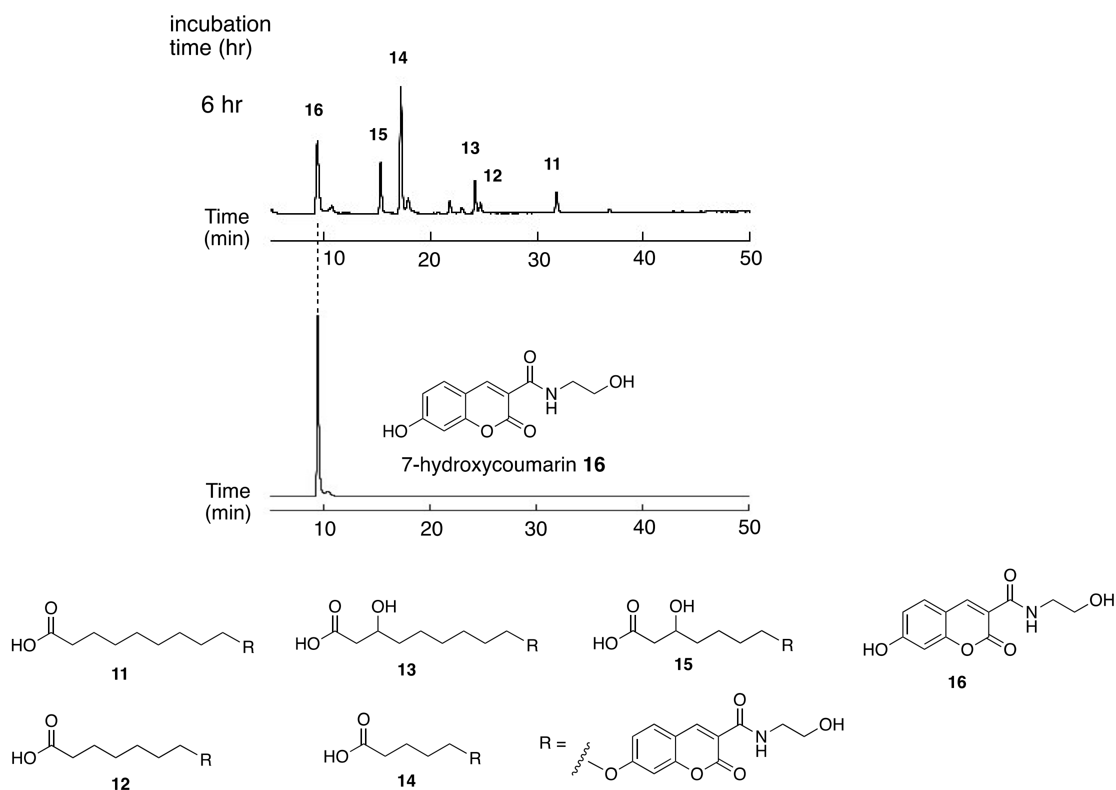


Figure S4. Confirmation of retention time of 7-hydroxycumarin **16** in HPLC analysis. The cells were treated with probe **10** (5 μM) for 1-6 h at 37 °C in HBS (+) buffer (pH = 7.4). Each peak was detected by UV absorbance (λ = 350 nm) and identified as compound **11**–**15** by ESI-TOF-MS analysis.

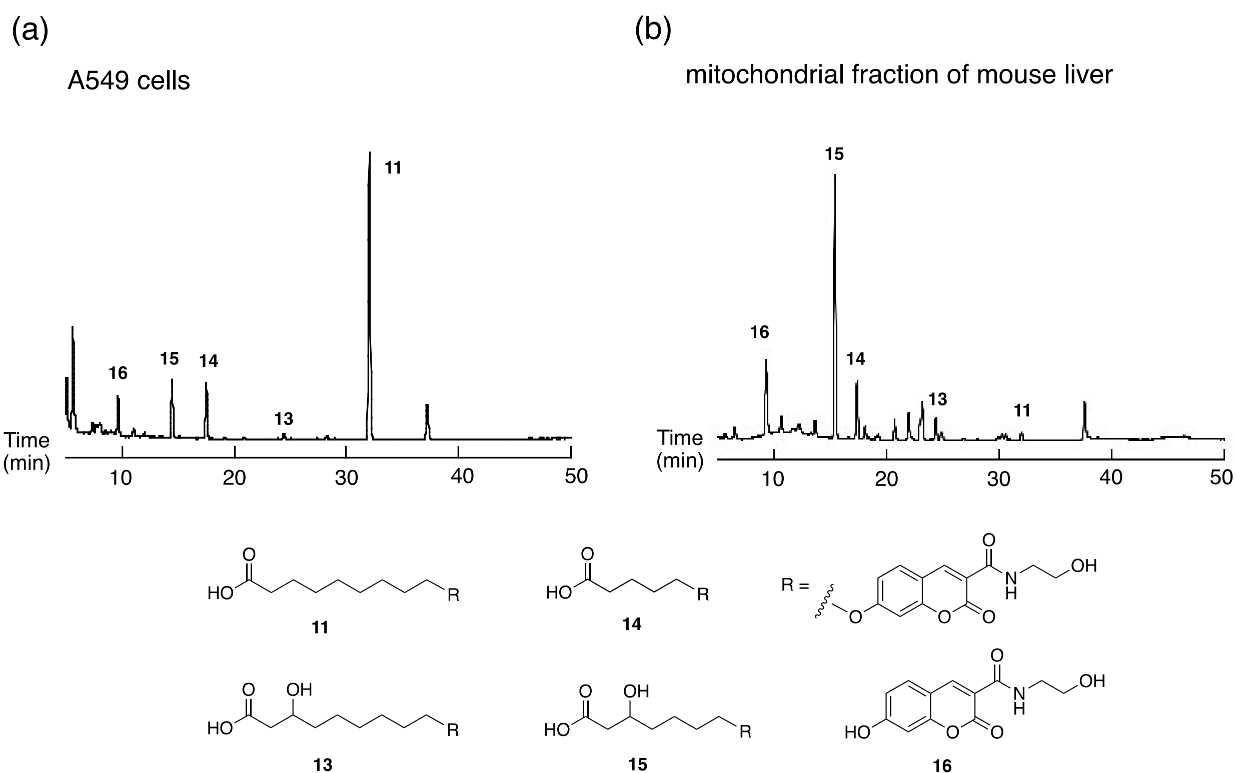


Figure S5. HPLC analysis of FAO metabolism of probe **10** in A549 cells (a) and mitochondrial fraction of mouse liver (b). The A549 cells were treated with probe **10** (5 μM) for 6 h at 37 °C in serum-free and phenol red-free DMEM. The mitochondrial fraction of mouse liver (100 mg/mL) was treated with probe **10** (20 μM) for 1h at 37 °C. Each peak was detected by UV absorbance (λ = 350 nm) and identified as compound **11–15** by ESI-TOF-MS analysis.

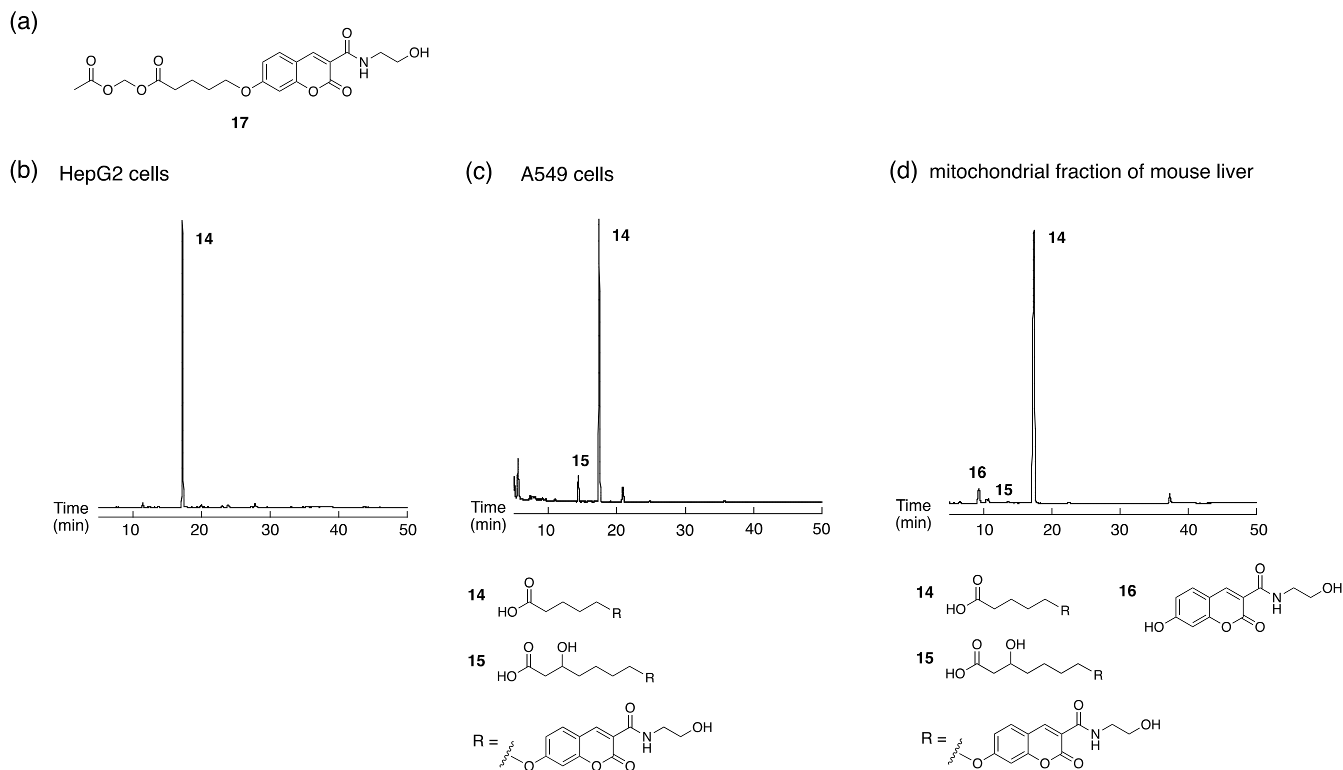
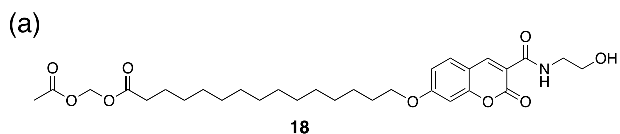


Figure S6. HPLC analysis of FAO metabolism of probe **17** (a) in live HepG2 (b), A549 (c) cells or the mitochondria fracution of mouse liver (d). The cells were treated with probe **17** (20 μM in HepG2 cells or 5 μM in A549 cells) for 6 h at 37 °C in serum-free and phenol red-free DMEM. The mitochondrial fraction of mouse liver (100 mg/mL) was treated with probe **17** (20 μM) for 1h at 37 °C. Each peak was detected by UV absorbance (λ = 350 nm).



(b) HepG2 cells

(c) A549 cells

(d) mitochondrial fraction of mouse liver

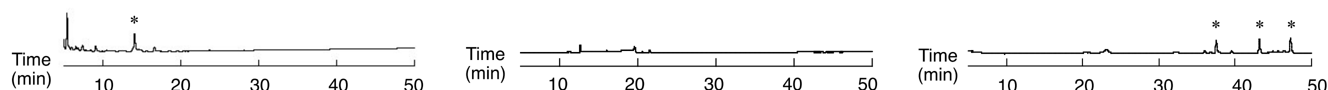


Figure S7. HPLC analysis of FAO metabolism of probe **18** (a) in live HepG2 (b), A549 (c) cells or the mitochondria fracution of mouse liver (d). The cells were treated with probe **18** (20 μM in HepG2 cells or 5 μM in A549 cells) for 6 h at 37 °C in serum-free and phenol red-free DMEM. Each peak was detected by UV absorbance (λ = 350 nm). The mitochondrial fraction of mouse liver (100 mg/mL) was treated with probe **18** (20 μM) for 1h at 37 °C. The peak marked with * was unidentified. Note that few peaks were not observed from the cells treated with **18** probably due to its high lipophilicity.

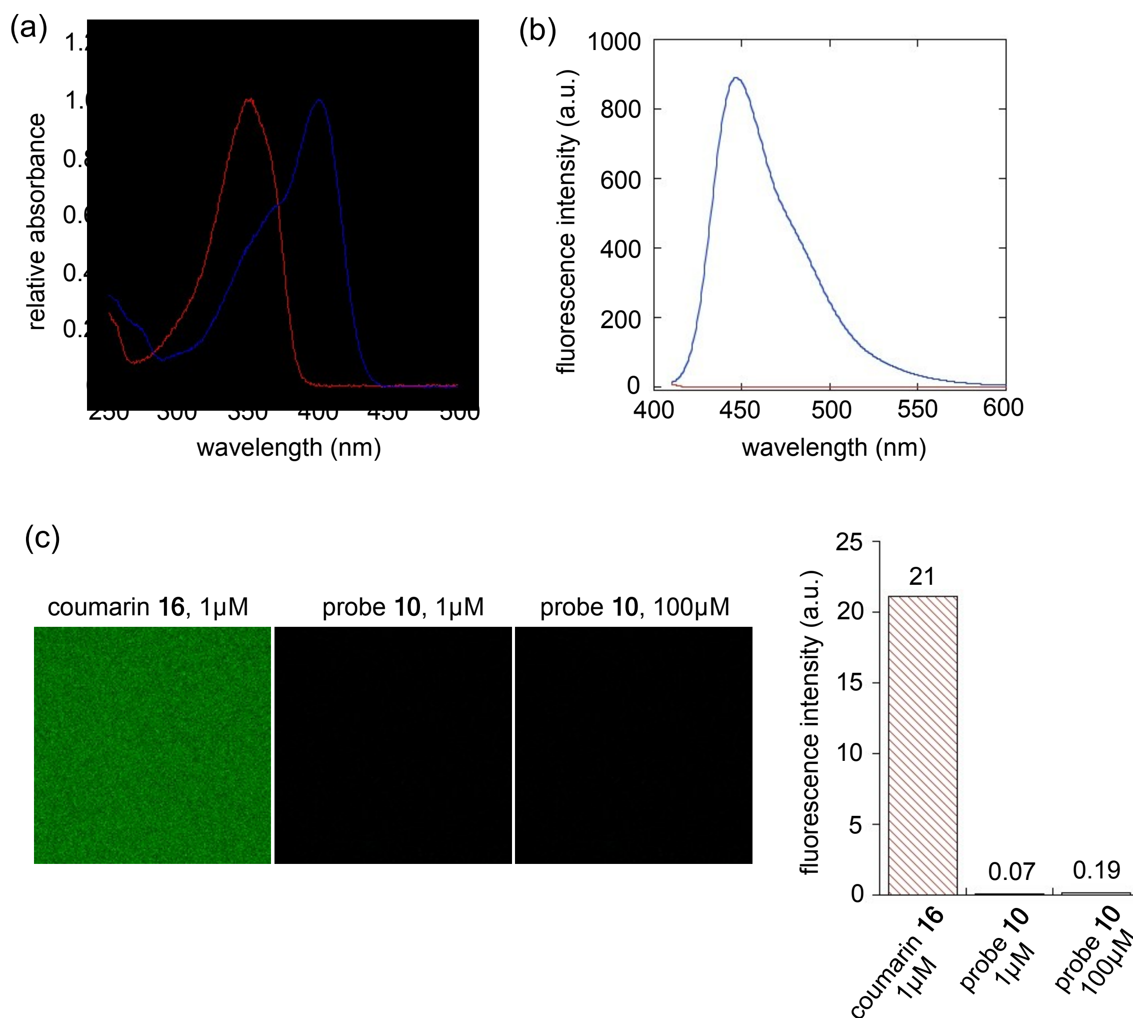


Figure S8. Evaluation of photophysical properties of probe **10** and 7-hydroxycoumarin **16**. (a, b) Absorbance and fluorescence spectra of **10** (red line) and **16** (blue line) in PBS buffer (pH = 7.4). The excitation wavelength was 405 nm in the fluorescence measurement. The extinction coefficient (ε) and fluorescence quantum yield (Φ) are summarized in Table S1. (c) Fluorescence detection of the aqueous solution of **10** and **16** by confocal microscopy. Conditions: [**10**] = 1 or 100 μM, [**16**] = 1 μM, HBS(+) buffer, λ_{ex} = 405 nm. Note that coumarin **16** is detectable with an at least 300-fold higher fluorescence intensity compared to **10** by confocal microscopy.

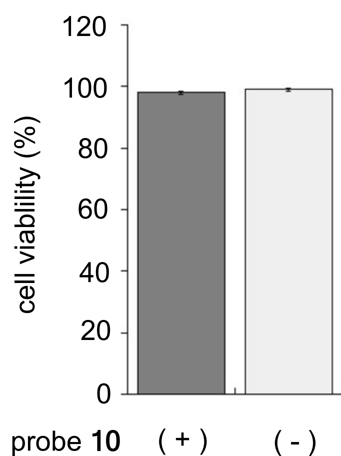
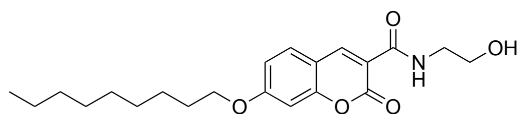
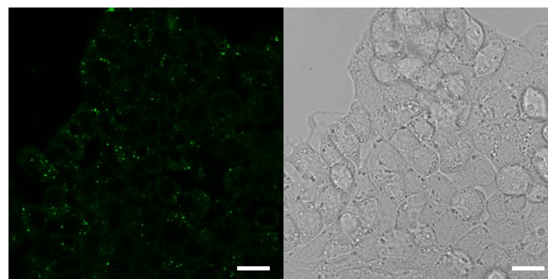


Figure S9. Evaluation of cell viability after treatment of HepG2 cells with probe **10**. The HepG2 cells were treated with or without probe **10** (5 μM) for 30 min at 37 °C in HBS(+) buffer (pH = 7.4). The cell viability was evaluated by the standard typan blue assay. Error bars represent standard deviation from the mean (n = 3).



19

probe **19**



DMSO

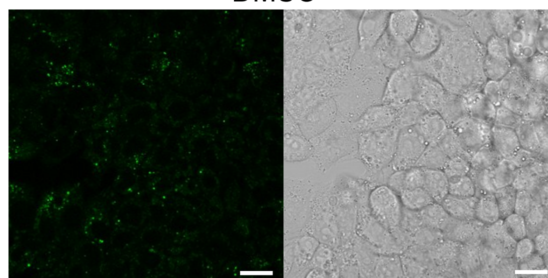


Figure S10. Evaluation of FAO metabolism of probe **19** in HepG2 cells. Fluorescence imaging of the cells upon incubation with **19** (5 μM) or DMSO (vehicle) in HBS (+) buffer (pH = 7.4) for 30 min at 37°C. Scale bar: 20 μm

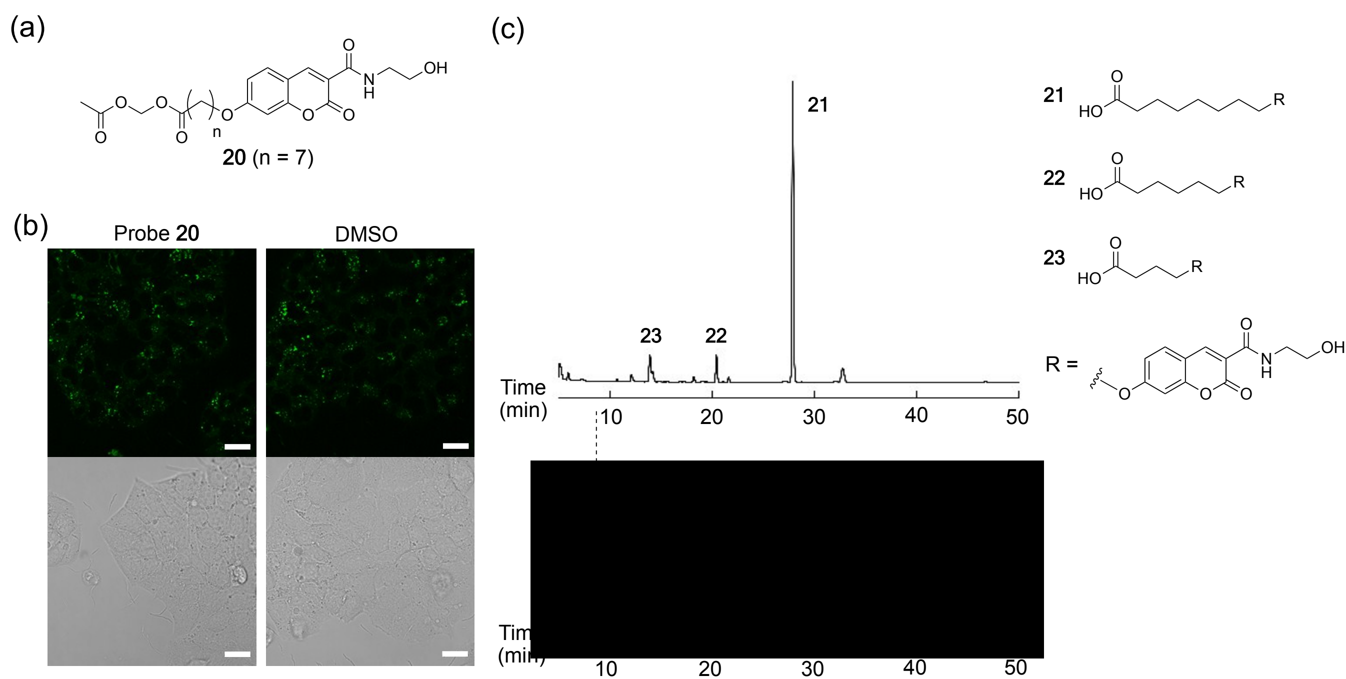


Figure S11. Evaluation of FAO metabolism of probe **20** in HepG2 cells. (a) Chemical struture of probe **20**. (b) Fluorescence imaging of the cells upon incubation with **20** (5 μM) or DMSO (vehicle) in HBS(+) buffer (pH = 7.4) for 30 min at 37°C. Scale bar: 20 μm (c) HPLC analysis of FAO metabolites derived from **22** in HepG2 cells. The cells were incubated with **20** (20 μM) in serum-free and phenol red-free DMEM for 6h at 37°C. Each peak was detected by UV absorbance (λ = 350 nm) and identified as compound **21–23** by ESI-TOF-MS analysis. Note that formation of hydroxycoumarin **16** was not detectable in the HPLC analysis.

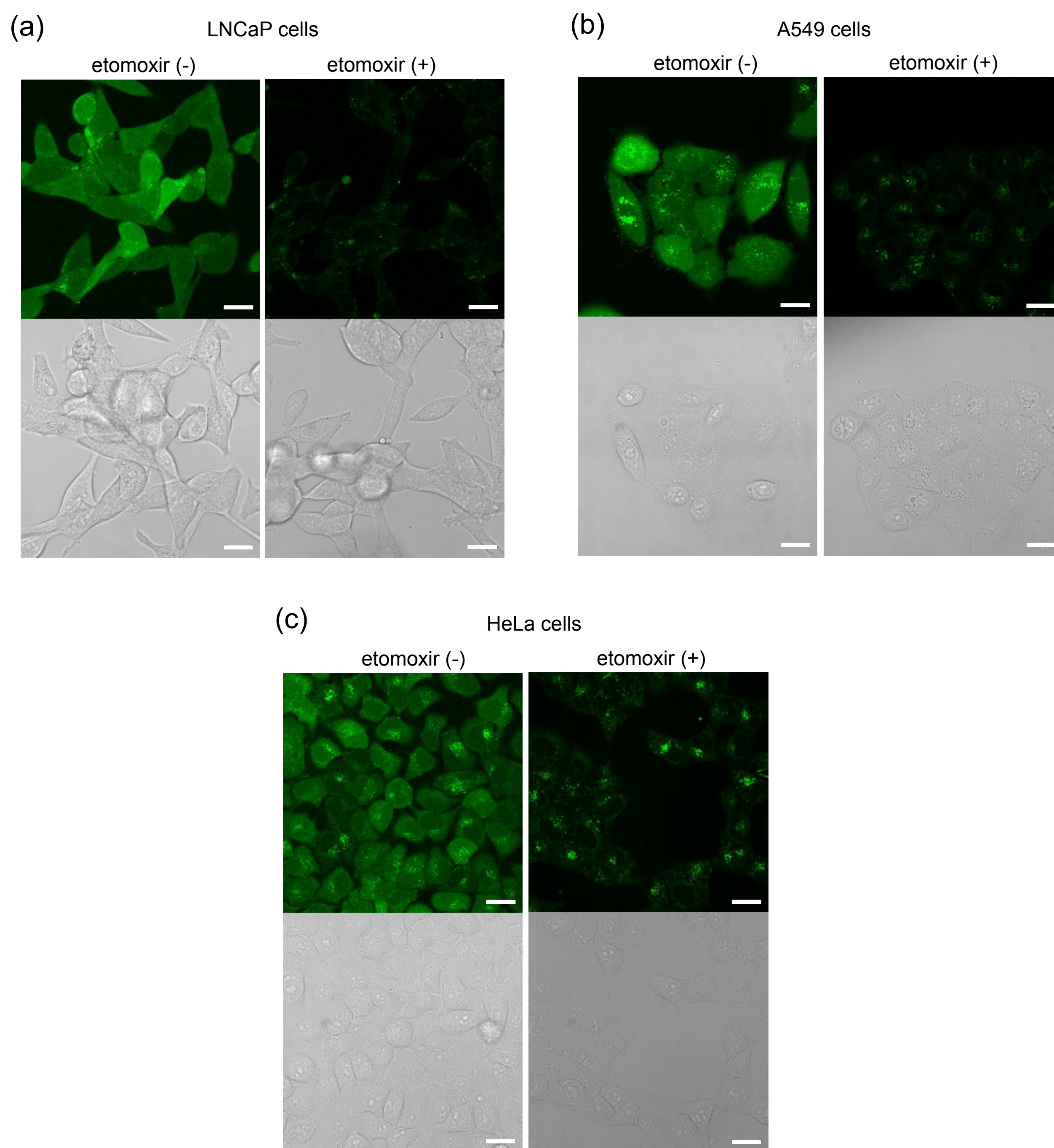


Figure S12. Fluorescence imaging of FAO activity in LNCaP, A549 and HeLa cells. The cells were incubation with **10** (20 μM) for 120 min in (a), **10** (5 μM) for 30 min in (b), and **10** (20 μM) for 120 min (c) in HBS (+) buffer (pH = 7.4) at 37°C. For the inhibition experiment, the cells were pre-treated with etomoxir (40 μM) for 3h in culture medium containing 10% FBS. Scale bar: 20 μm.

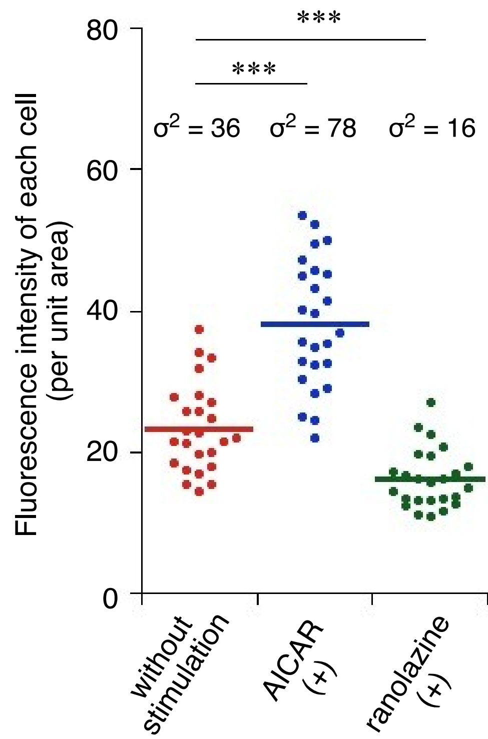


Figure S13. Statistical analysis (dot plot) of FAO activity in single HepG2 cells shown in Figure 3b (n = 25). The bar represents the mean value. σ^2 represents dispersion values. ***$P < 0.001$.

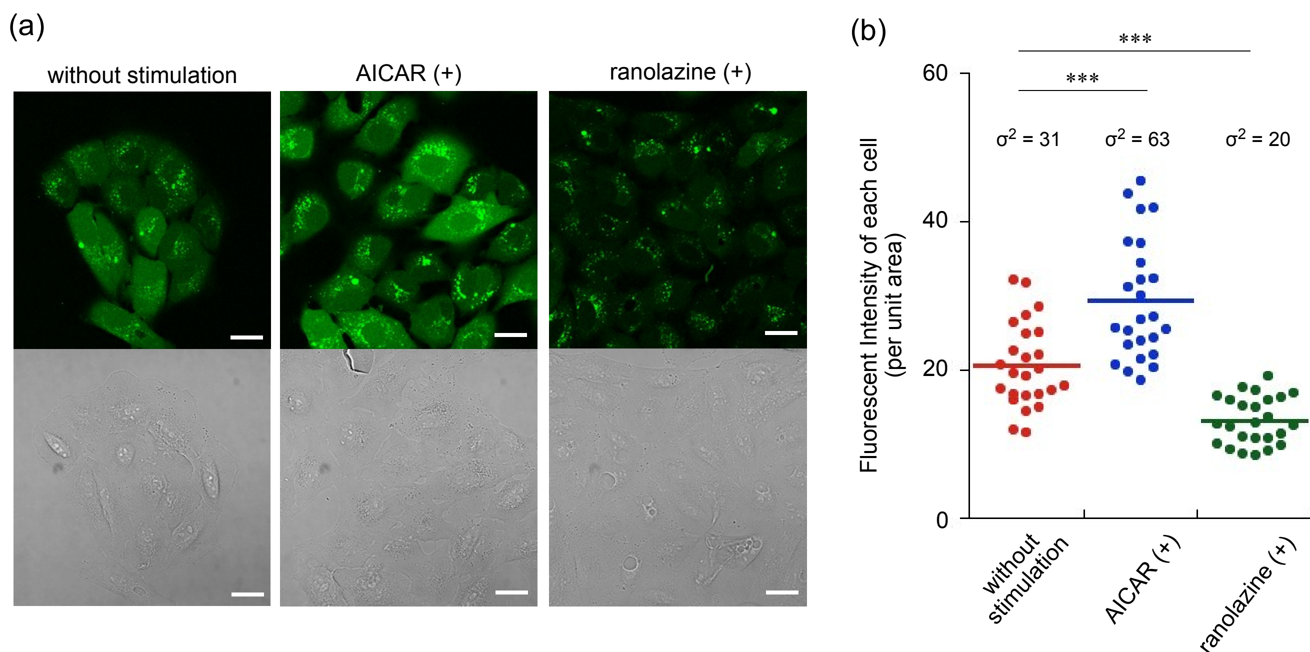


Figure S14. Evaluation of effect of the chemical modulators on FAO activity in A549 cells. (a) Fluorescence image of FAO activity in A549 cells upon treatment with AICAR or ranolazine. The cells were pre-treated with AICAR (200 μM) for 3h or ranolazine (200 μM) for 12h in DMEM (FBS+), followed by incubation with **10** (5 μM) in HBS (+) buffer (pH = 7.4) for 30 min at 37˚C. Scale bar: 20 μm. (b) Statistical analysis (dot plot) of fluorescence intensity (per unit area) of single A549 cells (n = 25). The bar represents the mean value. σ^2 represents dispersion values. ***$P < 0.001$.

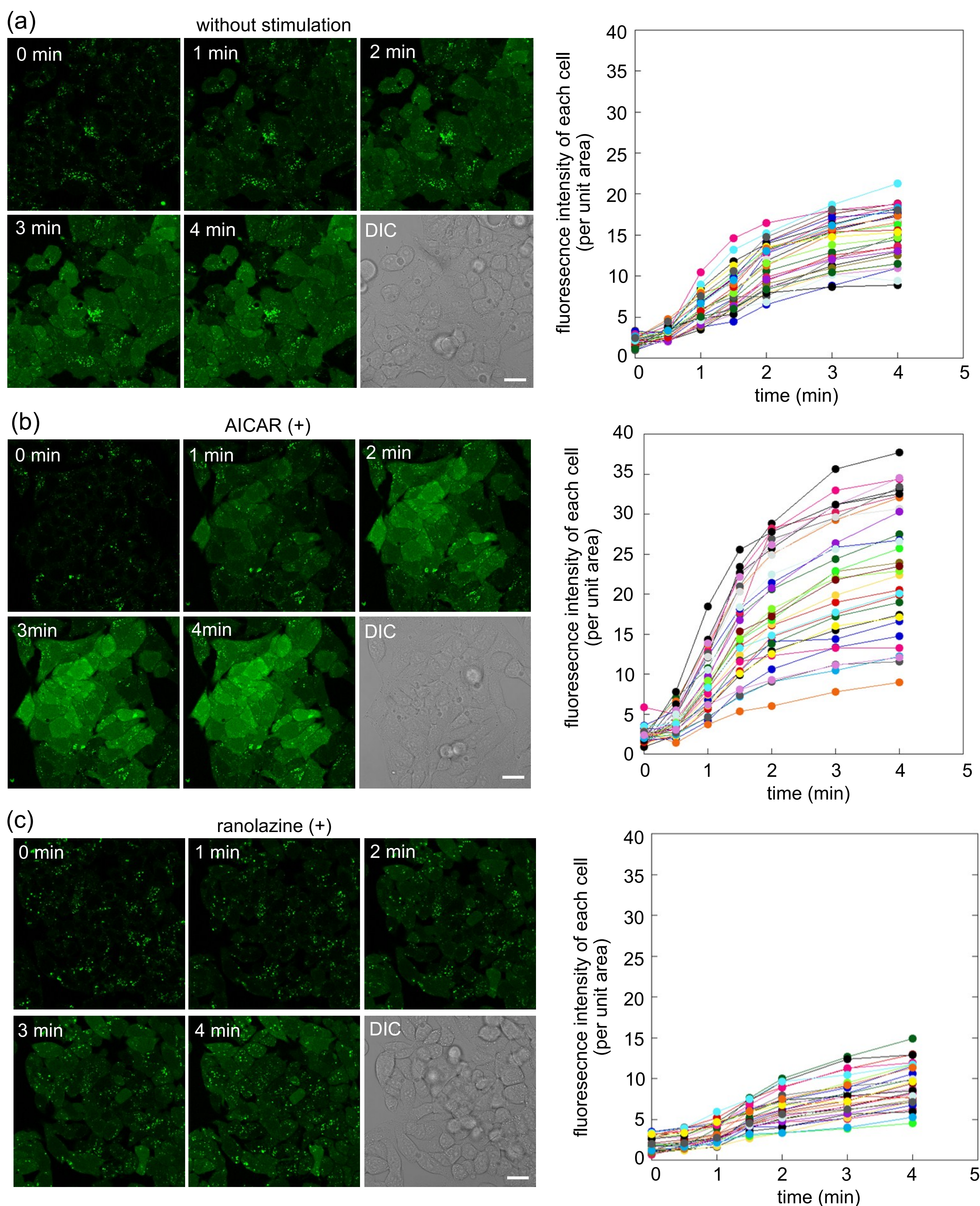
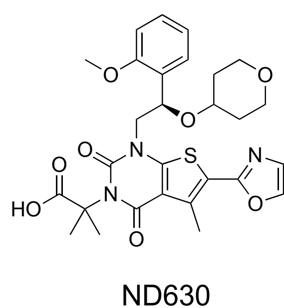
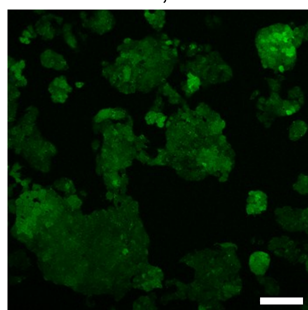


Figure S15. Analysis of FAO activity of each HepG2 cell base on initial rate of fluorescence increase (ΔF_{int} /min). The cells were treated with **10** (5 μM) in HBS (+) buffer (pH = 7.4) at 37 °C in the absence (a) and presence (b, c) of the chemical modulators. Fluorescence image (left) and time-course plot of fluorescence intensity in individual cells (n = 30) (right) are shown for each experiment. In the experiment of (b), the cells were incubated with AICAR (200 μM) for 3hr at 37 °C before the imaging. In the experiment of (c), the cells were incubated ranolazine (500 μM) for 12hr at 37 °C before the imaging. Scale bar: 20 μm.

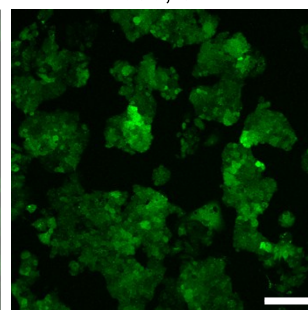
(a)



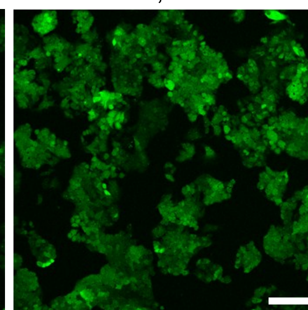
ND630, 0.2 nM



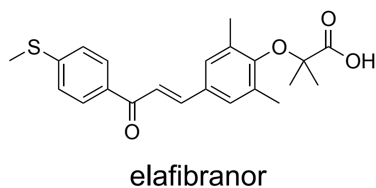
ND630, 30 nM



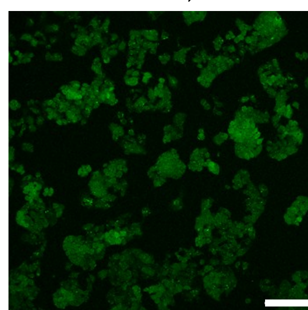
ND630, 100 nM



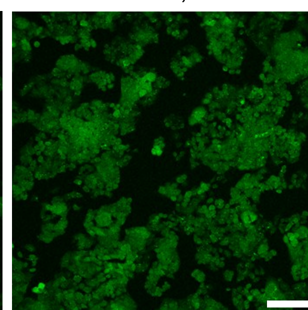
(b)



elafibranor, 20 nM



elafibranor, 500 nM



elafibranor, 1000 nM

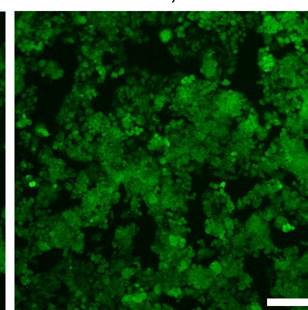


Figure S16. Concentration-dependent fluorescence change in HepG2 cells upon treatment with (a) ND630 and (b) elafibranor. HepG2 cells were pre-treated with ND-630 (a) for 4h at 37˚C or elafibranor (b) for 24h at 37˚C, followed by incubated with **10** (5 µM) for 30 min at 37˚C in HBS (+) buffer (pH = 7.4). Error bars represent standard deviation from the mean (n = 3). Scale bar: 150 µm.

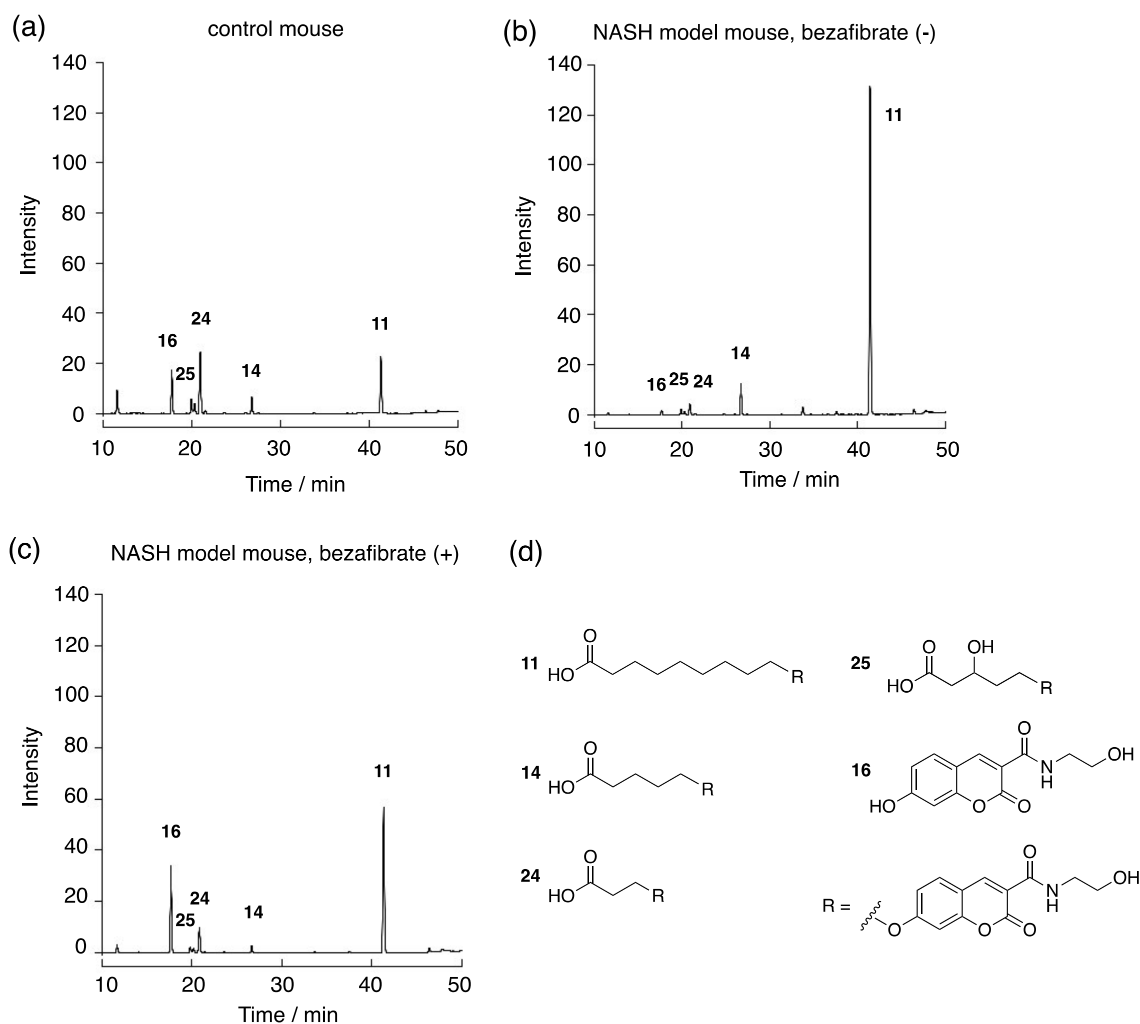


Figure S17. HPLC analysis of FAO metabolism of probe **10** in mouse hepatocytes. The hepatocytes isolated from (a) control mouse, (b) NASH model mouse and (c) NASH model mouse orally administered with bezafibrate was incubated with **10** (5 μM) in HBS (+) buffer (pH = 7.4) for 30 min at 37°C. (d) Chemical structures of the FAO metabolites derived from **10**. Each peak was detected by UV absorbance (λ = 350 nm) and identified by ESI-TOF-MS analysis.

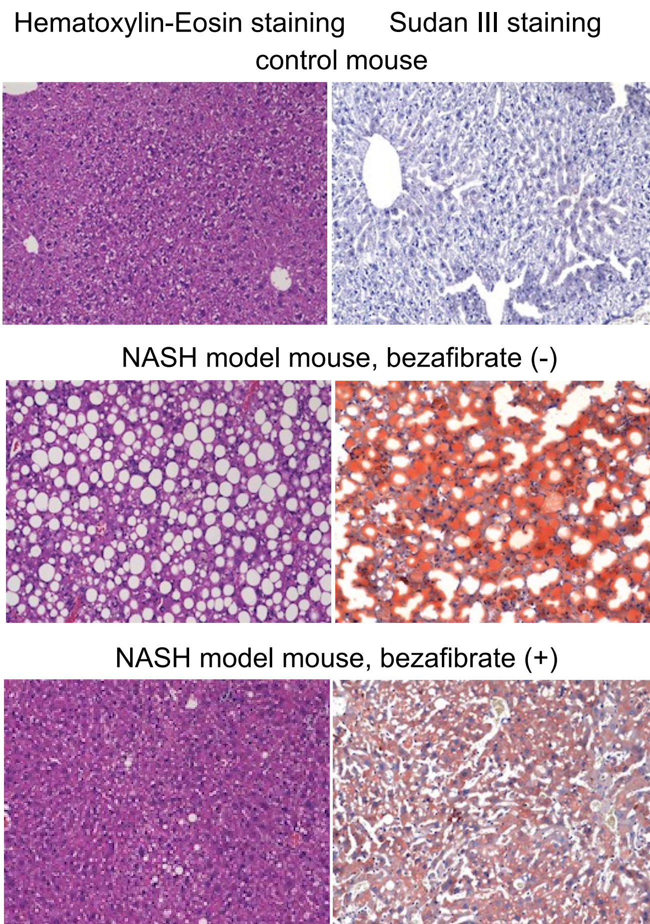


Figure S18. Hematoxylin-Eosin staining (left) and Sudan III staining (right) of the fixed liver tissue isolated from control mouse, NASH model mouse, and NASH model mouse orally administered with bezafibrate.

Experimental Details

Cell Culture

HepG2, A549 and HeLa cells were cultured in high-glucose Dulbecco's Modified Eagle medium (DMEM, 4.5 g of glucose/L, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 μg/mL) under humidified atmosphere of 5% CO_2 in air. LNCaP cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% FBS, penicillin (100 units/mL) and streptomycin (100 μg/mL) under humidified atmosphere of 5% CO_2 in air. Subculture was performed every 3-4 days from subconfluent (~ 80%) cultures using trypsin-EDTA solution.

HPLC analysis of probe metabolites in HepG2 cells and hepatocyte isolated from mouse

HepG2 cells ($5x10^5$ cells) were cultured on 6 cm dish (Falcon) for 2 days at 37°C in CO_2 incubator. After washing with serum-free and phenol red-free DMEM (Gibco) twice, the cells were incubated with each probe (5 or 20 μM) for 6 h at 37°C in 2 mL of serum-free and phenol red-free DMEM or HBS (+) buffer (pH = 7.4). After collecting the medium, the cells were lysed by sonication in 500 μL of PBS (-) buffer in an ice bath. After mixing with 500 μL of CH_3CN, insoluble stuff was removed by centrifugation (13,500 rpm, 10 min). The combined solution of the cell lysate and the medium was subjected to HPLC analysis (YMC-Triart C18, 250×10 mmI.D., Flow rate: 1.0 mL/min, mobile phase gradient: CH_3CN (0.1% TFA) / H_2O (0.1% TFA) = 20/80 → 70/30 linear gradient over 50 min, Detection wavelength: 320 nm for probe **3**, 350 nm for probe **10**, 370 nm for probe **2**, 520 nm for probe **1**). Compound identification of each peak was perfomed with ESI-TOF-MS (Bruker micrOTOF II)

For HPLC analysis of probe metabolites in hepatocyte isolated from mouse, the cells ($1x10^6$ cells) were cultured on 3.5 cm dish (Falcon) for 4h at 37°C in CO_2 incubator. After washing with HBS (+) (20 mM HEPES, 107 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 11.5 mM glucose, adjusted to pH 7.4 with NaOH) twice, the cells were incubated with probe **10** (5 μM) for 30 min at 37°C in 2 mL of HBS(+). The lysate sample was prepared as described above and subjected to HPLC analysis (mobile phase gradient: CH_3CN (0.1% TFA) / H_2O (0.1% TFA) = 10/90 → 60/40 linear gradient over 50 min, Detection wavelength: 350 nm). Compound identification of each peak was perfomed with ESI-TOF-MS (Bruker micrOTOF II).

Evaluation of probe metabolization in mitochondria isolated from mouse liver.

Mitochondria were isolated from mouse liver according to the reported method.[S1] The mitochondria pellet (50 mg/mL) was diluted with 1000 μL of reaction buffer (20 mM HEPES, 1 mM EGTA, 100 mM KCl, 5 mM KH_2PO_4, 10 mM $MgCl_2$, 25 mM sucrose, 2 mM carnitine, 5 mM ATP, 10 mg/ml BSA, 1.4 mM DTT, 0.13 mM CoA, pH = 7.4) and incubated with each probe (deprotection form of terminal carboxyl group, 20 μM) for 1h at 37°C. After lysis of mitochondria by addition of 400 μL of RIPA buffer, the solution (200 μL) was mixed with methanol (200 μL) and insoluble stuff was removed by centrifugation (13,500 rpm, 10 min). The supernatant was subjected to

HPLC analysis (YMC-Triart C18, 250×10 mmI.D., Flow rate: 1.0 mL/min, mobile phase gradient: CH_3CN (0.1% TFA) / H_2O (0.1% TFA) = 20/80 → 70/30 linear gradient over 50 min, Detection wavelength: 350 nm)

Evaluation of fluorescence properties of probe 10 and 7-hydroxycoumarin 16

Molar extinction coefficients (ε, M^{-1}•cm^{-1}) of **10** and **16** were determined in the mixed solvent system (50 mM HEPES buffer (pH = 7.2) : methanol = 1 : 1) and 50 mM HEPES buffer (pH = 7.2), respectively. Fluorescence quantum yields (Φ) of **10** and **16** were determined in the same soblent systems using quinine sulfate (Φ = 0.55) as a fluorescence quantum yield standard.

Fluorescence detection of FAO activity in living cells

HepG2, A549, HeLa or LNCaP cells ($1x10^5$ cells) were cultured on 3.5 cm glass-base dish (Iwaki) for 2-3 days at 37°C in CO_2 incubator. The cells were washed with HBS (+) buffer twice and incubated with **10** (5 μM) for 30 min in HepG2 and A549 cells, **10** (20 μM) for 2h in HeLa and LNCaP cells, respectively, in HBS(+) buffer (pH = 7.4) at 37°C. In control experiments, the cells were pretreated with etomoxir (40 μM) for 3h at 37°C in each medium, and then incubated with **10** as described above. The cells were then subjected to fluorescence imaging with confocal microscopy (TCS SP8, Leica microsystems) equipped with HyD detector. Fluorescence images were acquired using the 405 nm excitation derived from a semiconductor laser. For evaluation of the effects of AICAR and ranolazine, HepG2 or A549 cells were pretreated with AICAR (200 μM) for 3h, ranolazine (200 μM) for 12 h, respectively, at 37°C in DMEM (FBS(+)). The cells were then washed with HBS(+) buffer twice and incubated with **10** (5 μM) and each chemical modulator for 30 min at 37°C, followed by subjection to fluorescence imaging with confocal microscopy as described above. For fluorescence imaging of FAO activity in hepatocyte from mouse, the cells seeded on 3.5 cm collagen-coated glass-base dish (Iwaki) were washed with HBS(+) twice, incubated with **10** (5 μM) for 30 min at 37°C in HBS(+) and subjected to fluorescence imaging as described above.

Evaluation of FAO activity change by the metabolite-related drugs in HepG2 cells

HepG2 cells ($1x10^5$ cells) were cultured on 3.5 cm glass-based dish (Iwaki) for 2-3 days at 37°C in CO_2 incubator. The cells were then pretreated with ND630 (0-500 nM) for 4h, elafibranor (0-5000 nM) for 24h, respectively, in DMEM (FBS(+)). The cells were then washed with HBS(+) buffer twice and incubated with **10** (5 μM) and ND630/elafibranor in HBS(+) buffer (pH = 7.4) for 30 min at 37°C, followed by subjection to fluorescence imaging with confocal microscopy (TCS SP8, Leica microsystems) equipped with HyD detector. Averaged fluorescence intensity of the bulk population of cells was analyzed in 3 independent dishes. EC_{50} values of ND630 and elafibranor were calculated by curve-fitting analysis.

Evaluation of fluorescence change in individual cells

HepG2 cells ($1x10^5$ cells) were cultured on 3.5 cm glass-based dish (Iwaki) for 2-3 days at 37°C in CO_2 incubator. After washed with HBS (+) buffer twice and warmed to 37°C on the stage of confocal microscopy (TCS SP8, Leica microsystems), the cells were treated with probe **10** (5 μM) and subjected to fluorescence imaging at the same visual field. For evaluation of the effects of AICAR and ranolazine, HepG2 cells were pretreated with AICAR (200 μM) for 3h, ranolazine (200 μM) for 12 h, respectively, at 37°C in DMEM (FBS(+)), and subjected to fluorescence imaging as described above. Fluorescence intensity in individual cells was analyzed with ImageJ 1.52n.

Construction of NASH model mice

All animal experiments were approved by the Animal Experiment Committee of Kyushu University. Specific pathogen-free C57BL/6J male mice of 5 weeks of age were purchased from Japan SLC Inc. (Shizuoka, Japan) and were acclimated for 1 week before the start of treatments. Animals were maintained at 23 ± 3 °C with a 12:12 h light/dark cycle and fed with a commercial standard diet (#CE-2; CLEA Japan Inc., Shizuoka, Japan) and tap water ad libitum. Mice at 7 weeks of age was treated with the l-amino acid rodent diet with 60 kcal% fat and 0.1% methionine (#A06071302) or standard diet for 4weeks. The rodent diet (#A06071302) was purchased from EPS EKISHIN Co (Japan). At the end of each time point, mice were weighed and then killed by exsanguination under isoflurane anaesthesia. The liver was dispersed by collagenase type I (Sigma, St. Louis, MO, USA) perfusion method[S2]. Part of the liver tissue was fixed in formalin, and analyzed by sudan III stain.

Drug administration

Bezafibrate (Tokyo chemical industry co.,ltd.) was suspended in 0.05% carboxymethyl cellulose (CMC) solution. The NASH model mice were treated with bezafibrate (p.o., 400 mg/kg) or 0.05% CMC solution from starting time of 60 kcal% fat and 0.1% methionine feeding for 4 weeks.

Hepatocytes isolation and primary cell culture

Hepatocytes were isolated from control healthy mice or NASH model mice using a collagenase perfusion method[S2]. Viability of hepatocytes was assessed to be greater than 90% using trypan blue dye exclusion. Cells were plated on 3.5 cm dish (Falcon) or 3.5 cm collagen-coated glass-base dish (Iwaki) at a density of 1×10^6 cells per dish in Williams' medium (Sigma, St. Louis, MO, USA) containing 5% FBS, 0.1 μM insulin, 0.1 μM dexamethasone, and 2% penicillin/streptomycin and incubated in 37°C, 100% humidified environment (5% CO_2, 95% air). Following an attachment period of 4 hr, the culture medium was replaced with serum-free Williams' medium containing 0.1 μM insulin, 0.1 μM dexamethasone. The hepatocytes after isolation from liver at 4 hr were used to analyze the levels of beta-oxidation products.

Sudan III stain analysis

Small pieces of the remnant liver from NASH or control healthy mice were placed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight. The fixed tissue was stained with sudan III, followed by rinsing in 15% or 30% sucrose[S3]. The red-stained oil drops regions were examined using a computer-assisted image analyzer under Keyence BZ-9000 Fluorescence Microscope with a 20x objective.

Statistical analysis

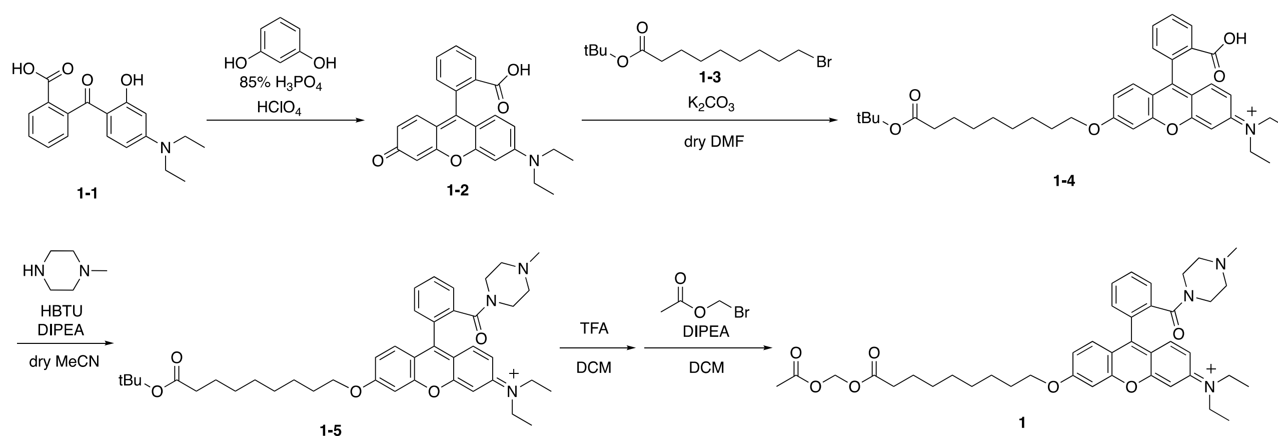
All statistical tests were performed using Microsoft Excel. Student's *t*-test (two-tailed distribution) was used to calculate P values. Number of experiments and samples were described in the figure legends. All data with three repetitive experiments or samples show the mean ± s.d. Nonlinear least-squares fitting was performed using KaleidaGraph Ver. 4.5 to obtain EC_{50} values of chemical modulator.

Synthesis and Characterization of the Compounds

General materials and methods for organic synthesis

Unless otherwise noted, chemical reagents were purchased from commercial suppliers (FUJIFILM Wako Pure Chemical Corporation, Tokyo Chemical Industry, Sigma-Aldrich) and used without further purification. Reactions were carried out under a positive atmosphere of nitrogen, unless otherwise stated. Reactions were monitored by thin layer chromatography (TLC) carried out on Merck TLC Silica gel 60 F_{254}, using shortwave UV light as the visualizing agent. ^{1}H NMR spectra were recorded using a Varian UNITY-400 (400 MHz) spectrometer or Bruker Avance III HD 500 MHz spectrometer and chemical shifts (δ, ppm) were referenced to residual solvent peak ($CDCl_3$: 7.26 ppm; MeOH-d_4: 3.31 ppm; DMSO-d_6: 2.50 ppm). ESI mass spectrometry was recorded using a MicroTOF II (Bruker Daltonics) spectrometer. HPLC purification was conducted with a HITACHI L-7000 series (Hitach).

Preparation of 1



Synthesis of 1-2

To a solution of **1-1** (1.00 g, 3.20 mmol) in H_3PO_4 85w% H_2O (5 mL) was added m-resorcinol (405 mg, 3.68 mmol). After stirred at 170 °C for 1h, the solution was cooled down to room temperature and $HClO_4$ (5 mL) was added. The mixture was stirred at 100 °C for 15 min and carefully quenched with sat. $NaHCO_3$ aq. After extracted with chloroform, the crude product was purified by column chromatography on SiO_2 (chloroform : methanol = 40:1 to 20:1) to give **1-2** (990 mg, 80%) as a red solid. ^{1}H-NMR (400 MHz, CD_3OD): δ 8.07 (1H, d, J = 8.0 Hz), 7.69-7.73 (2H, m), 7.25 (1H, d, J = 8.0 Hz), 6.83 (2H, d, J = 8.8 Hz), 6.69-6.74 (3H, m), 6.61 (1H, dd, J = 2.4, 8.8 Hz), 3.52 (4H, q, J = 7.2 Hz), 1.23 (6H, t, J = 7.6 Hz). ESI-TOF-MS m/z calcd. for $C_{24}H_{21}NO_4$ $[M+H]^+$ 388.1543, found 388.1549

Synthesis of 1-4

To a solution of **1-2** (50.0 mg, 0.130 mmol) and K_2CO_3 (53.4 mg, 0.387 mmol) in dry DMF (2 mL) was added **1-3**[S4] (57.1 mg, 0.195 mmol), and the mixture was stirred at 50 °C for 1h. After the removal of the solvent, the residue was diluted with chloroform and washed with sat. $NaHCO_3$ aq., water and brine. The mixture was purified by column chromatography on SiO_2 (chloroform : methanol = 40:1 to 20:1) to give **1-4** (59.2 mg, 76%) as a red solid. ^{1}H-NMR (400 MHz, CD_3OD): δ 8.25 (1H, d, J = 7.6 Hz), 7.41-7.84 (1H, m), 7.40 (1H, dd, J = 1.2, 6.4 Hz), 6.87-6.99 (2H, m), 6.85-6.86 (2H, m), 6.54 (1H, dd, J = 2.4, 9.6 Hz), 6.51 (1H, d, J = 2.0 Hz), 3.92 (2H, t, J = 6.0 Hz), 3.60 (4H, q, J = 7.8 Hz), 1.51-1.56 (2H, m), 1.44 (9H, s), 1.27 (6H, t, J = 7.8 Hz), 1.10-1.23 (6H, m), ESI-TOF-MS *m/z* calcd. for $C_{37}H_{46}NO_6$ $[M]^+$ 600.3320, found 600.3311

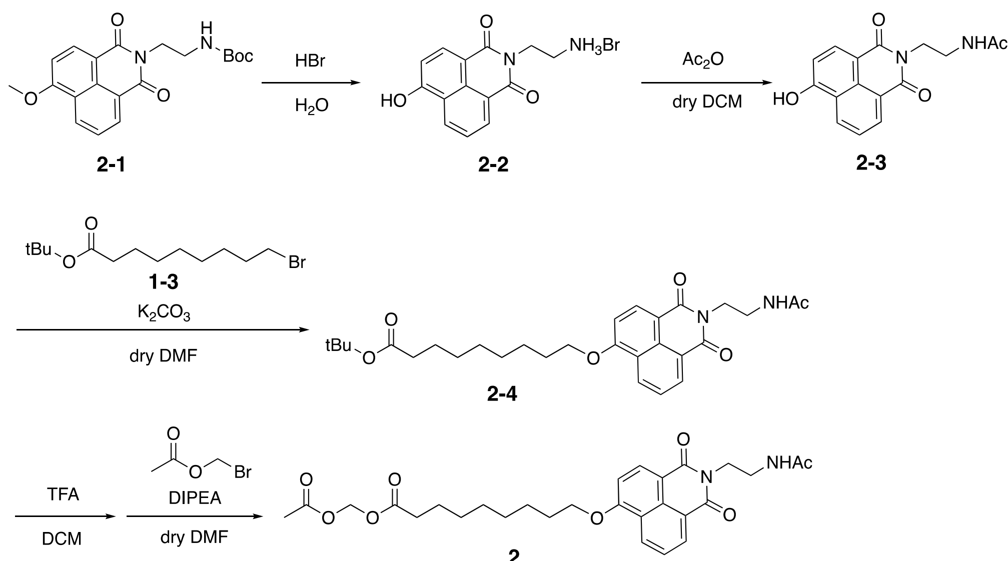
Synthesis of 1-5

To a solution of **1-4** (65.4 mg, 0.109 mmol), HBTU (61.9 mg, 0.163 mmol) and DIPEA (75.8 µL, 0.436 mmol) in dry CH_3CN (3 mL) was added N-methyl piperazine (75.8 µL, 0.436 mmol), and the mixture was stirred overnight at 50°C. After the removal of the solvent, the residue was diluted with chloroform and washed with sat. $NaHCO_3$ aq., water and brine. The mixture was purified by column chromatography on SiO_2 (chloroform : methanol = 20:1 to 10:1, 1% (v/v) of NH_3) to give **1-5** (6.3 mg, 8.5%) as a red oil. ^{1}H-NMR (400 MHz, CD_3OD): δ 8.32 (1H, dd, J = 1.2, 8.0), 7.87 (1H, dt, J = 1.6, 7.2 Hz), 7.82 (1H, dt, J = 1.6, 7.2 Hz), 7.44 (1H, dd, J = 1.6, 8.0 Hz), 7.20-7.25 (m, 3H), 7.12-7.18 (m, 2H), 7.04 (1H, d, J = 2.4 Hz), 3.91-3.98 (m, 2H), 3.82 (4H, t, J = 4.8 Hz), 3.73 (4H, q, J = 6.8 Hz), 2.80 (4H, t, J = 4.8 Hz), 2.50 (s, 3H), 2.20 (2H, t, J = 7.6 Hz), 1.44 (s, 9H), 1.24-1.35 (m, 14H), 1.07-1.18 (2H, m), 0.88-0.99 (2H, m). ESI-TOF-MS *m/z* calcd. for $C_{42}H_{56}N_3O_5$ $[M]^+$ 682.4214, found 682.4230

Synthesis of 1

To a solution of **1-5** (6.3 mg, 9.2 µmol) in DCM (1 mL) was added TFA (0.3 mL), and the solution was stirred for 1h at room temperature. After removal of the solvent by evaporation, the residue was diluted with dry DMF (1 mL). To the solution were added DIPEA (16.0 µL, 92.2 µmol) and bromomethyl acetate (2.6 µL, 27.7 µmol), and the mixture was stirred overnight at room temperature. After the removal of the solvent, the residue was purified by reverse-phase HPLC (YMC-Triart C18, 250×10 mmI.D., Flow rate: 3.0 mL/min, mobile phase gradient: CH_3CN (0.1% TFA) / H_2O (0.1% TFA) = 30/70 → 70/30, linear gradient over 60 min) to give **1** (1.3 mg, 15 % in 2 steps) as a red solid. ^{1}H-NMR (500 MHz, CD_3OD): δ 8.33 (1H, dd, J = 1.5, 7.5 Hz), 7.83-7.90 (2H, m), 7.45 (1H, dd, J = 1.5, 7.5 Hz), 7.34 (1H, d, J = 2.0 Hz), 7.19-7.27 (4H, m), 7.08 (1H, d, J = 2.0 Hz), 5.74 (2H, s), 3.89-4.00 (4H, m), 3.74-3.78 (4H, m), 3.00 (s, 3H), 2.27 (2H, t, J = 7.5 Hz), 2.11 (3H, s), 1.54-1.60 (2H, m), 1.33-1.36 (6H, m), 1.23-1.31 (4H, m), 1.11 (4H, m), 0.96-1.02 (2H, m). ESI-TOF-MS *m/z* calcd. for $C_{41}H_{52}N_3O_7$ $[M]^+$ 698.3800, found 698.3810

Preparation of 2



Synthesis of 2-2

2-1[S5] (100 mg, 0.270 mmol) was dissolved in 48% HBr in water (10 mL) and the solution was stirred overnight at 100 °C. After cooled down to room temperature, the precipitation was collected by filtration and washed with water to give **2-2** (75.0 mg, 82%) as a yellow solid. ^{1}H-NMR (400 MHz, CD_3OD): δ 8.66 (1H, dd, *J* = 1.2, 8.4 Hz), 8.59 (1H, dd, *J* = 1.2, 8.0 Hz), 8.46 (1H, d, *J* = 8.0 Hz), 7.74 (1H, t, *J* = 7.2 Hz), 7.07 (1H, d, *J* = 8.4 Hz), 4.47 (2H, t, *J* = 6.0 Hz), 3.30-3.35 (2H, m). ESI-TOF-MS *m/z* calcd. for $C_{14}H_{13}N_2O_3$ $[M+H]^+$ 257.0926, found 257.0938

Synthesis of 2-3

To a solution of **2-2** (32.4 mg, 0.126 mmol) and DIPEA (43.8 µL, 0.252 µmol) in dry DCM (1 mL) was slowly added Ac_2O (13.1 µL, 0.139 mmol) in an ice bath, and the mixture was stirred for 2h at 0 °C. The solution was diluted with $CHCl_3$ and washed with water and brine. The mixture was purified by column chromatography on SiO_2 (chloroform : methanol = 20:1→5:1) to give **2-3** (10.0 mg, 27%) as a yellow solid. ^{1}H-NMR (400 MHz, CD_3OD): δ 8.61 (1H, dd, *J* = 1.2, 8.0 Hz), 8.41 (1H, d, *J* = 8.4 Hz), 7.71 (1H, dd, *J* = 6.8, 8.0 Hz), 7.06 (1H, d, *J* = 8.0 Hz), 4.30 (2H, t, *J* = 6.0 Hz), 3.53 (2H, t, *J* = 6.0 Hz), 1.83 (3H, s), ESI-TOF-MS *m/z* calcd. for $C_{16}H_{15}N_2O_5$ $[M+H]^+$ 299.1032, found 299.1051

Synthesis of 2-4

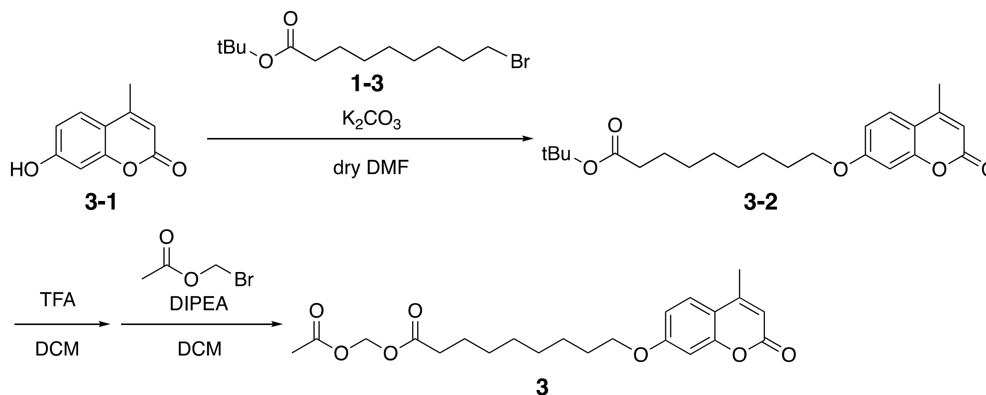
To a solution of **2-3** (5.0 mg, 16.8 µmol) and K_2CO_3 (7.0 mg, 50.4 µmol) in dry DMF (1 mL) was added **11-2** (5.9 mg, 20.1 µmol), and the mixture was stirred overnight at 50 °C. After quenching the reaction by addition of water, the crude product was extracted by ethyl acetate. The combined organic layer was washed with brine. After removal of the solvent by evaporation under reduced pressure, the residue was purified by column

chromatography on SiO_2 (chloroform : methanol = 40:1) to give **2-4** (8.8 mg, quant) as a yellow solid. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.59-8.62 (2H, m), 8.55 (1H, d, J = 8.4 Hz), 7.72 (1H, t, J = 8.4 Hz), 7.04 (1H, d, J = 8.0 Hz), 4.40 (2H, t, J = 6.8 Hz), 4.28 (2H, t, J = 6.8 Hz), 3.62-3.66 (2H, m), 2.21 (2H, t, J = 7.2 Hz), 1.94-2.02 (2H, m), 1.91 (3H, s), 1.44 (9H, s), 1.26-1.40 (10H, m), ESI-TOF-MS *m/z* calcd. for $C_{16}H_{15}N_2O_4$ $[M+H]^+$ 511.2808, found 511.2822

Synthesis of 2

To a solution of **2-4** (8.8 mg, 17.2 μmol) in DCM (2 mL) was added TFA (0.5 mL), and the solution was stirred for 2h at room temperature. After removal of the solvent by evaporation under reduced pressure, the residue was diluted with dry DMF (1 mL). To the solution were added DIPEA (30.0 μL, 172 μmol) and bromomethyl acetate (4.8 μL, 51.6 μmol), and the mixture was stirred overnight at room temperature. After the removal of the solvent, the residue was purified by reverse-phase HPLC (YMC-Triart C18, 250×10 mmI.D., Flow rate: 3.0 mL/min, mobile phase gradient: CH_3CN (0.1% TFA) / H_2O (0.1% TFA) = 30/70 → 70/30, linear gradient over 60 min) to give **2** (3.9 mg, 43 % in 2 steps). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.59-8.63 (2H, m), 8.55 (1H, d, J = 8.8 Hz), 7.72 (1H, t, J = 7.2 Hz), 5.74 (2H, s), 5.26-5.37 (2H, m), 4.40 (2H, t, J = 6.0 Hz), 4.28 (2H, t, J = 6.4 Hz), 3.62-3.67 (2H, m), 2.34-2.39 (2H, m), 2.11 (3H, s), 1.91 (3H, s), ESI-TOF-HRMS *m/z* calcd. for $C_{28}H_{34}N_2O_8Na$ $[M+H]^+$ 549.2207, found 549.2221

Preparation of 3



Synthesis of 3-2

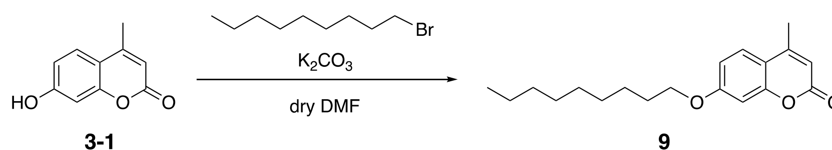
To a solution of **3-1**[S6] (15.0 mg, 85.0 μmol) and K_2CO_3 (35.2 mg, 255 μmol) in dry DMF (1 mL) was added **1-3** (30.0 mg, 102 μmol), and the mixture was stirred overnight at 50 °C. After quenching the reaction by the addition of water, the crude product was extracted with ethyl acetate. The combined organic layer was washed with brine. After removal of the solvent by evaporation under reduced presser, the residue was purified by

column chromatography on SiO_2 (hexane : ethyl acetate = 10:1 to 5:1) to give **3-2** (28.1 mg, 85%) as colorless oil. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.48 (1H, d, *J* = 8.4 Hz), 6.85 (1H, dd, *J* = 2.4, 6.4 Hz), 6.81 (1H, d, *J* = 2.4 Hz), 6.12 (1H, d, *J* = 1.2 Hz), 4.00 (1H, t, *J* = 6.4 Hz), 2.93 (3H, s), 2.23 (2H, t, *J* = 7.6 Hz), 1.77-1.84 (2H, m), 1.57-1.60 (2H, m), 1.44 (9H, s), 1.26-1.35 (8H, m), ESI-TOF-MS *m/z* calcd. for $C_{23}H_{35}O_5$ $[M+H]^+$ 389.2328, found 389.2341

Synthesis of 3

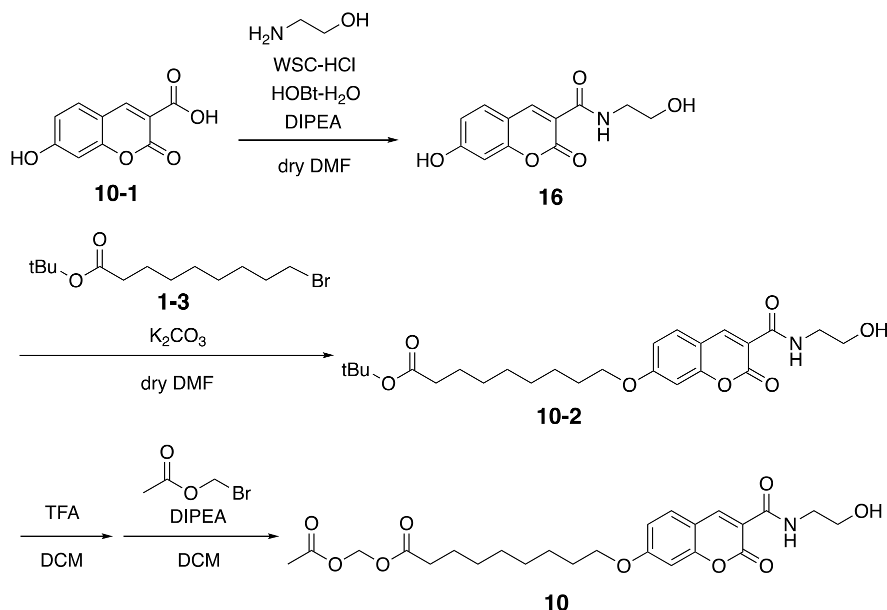
To a solution of **3-2** (28.1 mg, 72.3 μmol) in DCM (2 mL) was added TFA (0.5 mL), and the solution was stirred for 2h at room temperature. After removal of the solvent by evaporation, the residue was diluted with 1 mL of dry DMF. To the solution were added DIPEA (62.8 μL, 362 μmol) and bromomethyl acetate (33.2 μL, 217 μmol), and the mixture was stirred overnight at room temperature. After the removal of the solvent, the residue was purified by reverse-phase HPLC (YMC-Triart C18, 250×10 mmI.D. mobile phase gradient: CH_3CN (0.1% TFA) / H_2O (0.1% TFA) = 30/70 → 70/30, linear gradient over 60 min) to give **3** (4.8 mg, 16 % in 2 steps). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.48 (1H, d, *J* = 9.2 Hz), 6.85 (1H, dd, *J* = 2.8, 9.2 Hz), 6.81 (1H, d, *J* = 2.4 Hz), 6.13 (1H, s), 5.74 (2H, s), 4.01 (2H, t, *J* = 6.8 Hz), 2.40 (3H, s), 2.37 (2H, t, *J* = 7.2 Hz), 2.11 (3H, s), 1.79-1.85 (2H, m), 1.63-1.67 (2H, m), 1.45-1.47 (2H, m), 1.31-1.40 (6H, m), ESI-TOF-HRMS *m/z* calcd. for $C_{22}H_{28}NO_7Na$ $[M+Na]^+$ 427.1727, found 427.1715

Preparation of 9



To a solution of **3-1** (50.0 mg, 284 μmol) and K_2CO_3 (117 mg, 851 μmol) in 3 mL of dry DMF was added 1-bromononane (54.1 μL, 284 μmol), and the mixture was stirred for 6h at 50 °C. After evaporation of the solvent under reduced pressure, the crude product was diluted with Ethyl acetate and washed with brine. After removal of the solvent by evaporation under reduced presser, the residue was purified by column chromatography on SiO_2 (hexane : ethyl acetate = 5:1) to give **9** (70.1 mg, 82%) as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.48 (1H, d, *J* = 8.8 Hz), 6.85 (1H, dd, *J* = 2.4, 8.8 Hz), 6.80 (1H, d, *J* = 2.4 Hz), 6.12 (s, 1H), 4.01 (2H, t, *J* = 6.4 Hz), 2.39 (3H, s), 1.78-1.85 (2H, m), 1.43-1.50 (2H, m), 1.29-1.38 (10H, m), 0.89 (3H, t, *J* = 6.8 Hz). ESI-TOF-HRMS *m/z* calcd. for $C_{19}H_{27}O_3$ $[M+H]^+$ 303.1955, found 303.1944

Preparation of 10



Synthesis of 16

To a solution of **10-1**[S5] (100 mg, 485 μmol), HOBt-H_2O (89.0 mg, 582 μmol), WSCI-HCl (111 mg, 582 μmol) and DIPEA (337 μL, 1.94 mmol) in dry DMF (3 mL) was added ethanolamine (35.2 μL. 582 μmol) and the mixture was stirred overnight at room temperature. After removal of the solvent by evaporation under reduced pressure, the residue was washed with the solution of chloroform and methanol (1:1) to give **16** (57.1 mg, 41%) as a yellow solid. ^{1}H-NMR (400 MHz, CD_3OD): δ 8.77 (1H, s), 7.66 (1H, d, J = 8.8 Hz), 6.87 (1H, dd, J = 2.0, 8.8 Hz), 6.76 (1H, d, J = 2.4 Hz), 3.71 (2H, t, J = 5.6 Hz), 3.54 (2H, t, J = 6.4 Hz). ESI-TOF-MS *m/z* calcd. for $C_{12}H_{12}NO_5$ $[M+H]^+$ 250.0715, found 250.0727

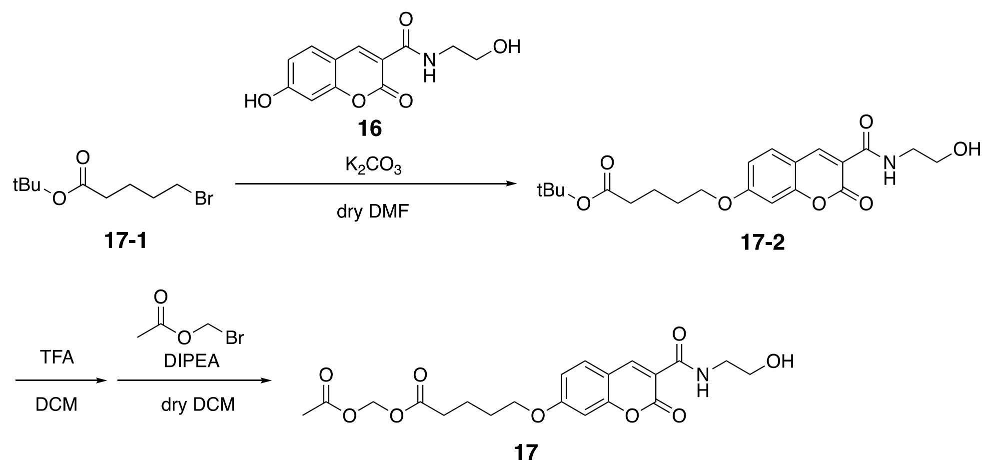
Synthesis of 10-2

To a solution of **16** (20.0 mg, 80.3 μmol) and K_2CO_3 (33.2 mg, 241 μmol) in dry DMF (2 mL) was added **1-3** (35.3 mg, 120 μmol) and stirred for 5h at 50 °C. After removal of the solvent by evaporation under reduced pressure, the residue was diluted with chloroform and the organic layer was washed with brine. The crude product was purified by column chromatography on SiO_2 (chloroform : methanol = 50 : 1) to give **10-2** (29.0 mg, 78%) as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 9.12 (1H, t, J = 5.6 Hz), 8.84 (1H, s), 7.57 (1H, d, J = 8.8 Hz), 6.93 (1H, dd, J = 2.4 , 8.8 Hz), 6.85 (1H, d, J = 2.4 Hz), 4.05 (2H, t, J = 6.8 Hz), 3.83-3.85 (2H, m), 3.62-3.66 (2H, m), 2.21 (2H, t, J = 7.6 Hz), 1.44 (9H, s), 1.31-1.41 (12H, m), ESI-TOF-MS *m/z* calcd. for $C_{25}H_{36}NO_7$ $[M+H]^+$ 462.2492, found 462.2483

Synthesis of 10

To a solution of **10-2** (29.0 mg, 62.8 μmol) in DCM (3 mL) was added TFA (1 mL) and the mixture was stirred at room temperature for 1h. After removal of the solvent by evaporation, the residue was diluted with dry DMF (1 mL). To the solution were added DIPEA (109 μL, 628 μmol) and bromomethyl acetate (17.6 μL, 188 μmol), and the mixture was stirred at 50 °C for 14h. After removal of the solvent by evaporation under reduced presser, the mixture was diluted with chloroform and washed with brine. After removal of the reaction by evaporation, the residue was purified by column chromatography on SiO_2 (Chloroform : Methanol = 50 : 1), followed by further purification with HPLC (YMC-Triart C18, 250×10 mmI.D., Flow rate; 3.0 mL/min, detection UV (220 nm), mobile phase gradient : CH_3CN (0.1% TFA) / H_2O (0.1% TFA) = 55/45 → 85/15, linear gradient over 40 min) to give **10** (3.1 mg, 25%) as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 9.10 (1H, t, *J* = 5.6 Hz), 8.84 (1H, s), 7.57 (1H, d, *J* = 8.8 Hz), 6.93 (1H, dd, *J* = 2.4, 9.2 Hz), 6.85 (1H, d, *J* = 2.4 Hz), 5.74 (2H, s), 4.05 (2H, t, *J* = 6.4 Hz), 3.84 (2H, t, *J* = 5.2 Hz), 3.62-3.66 (2H, m), 2.36 (2H, t, *J* = 7.6 Hz), 2.11 (3H, s), 1.79-1.85 (2H, m), 1.47-1.67 (4H, m), 1.32 -1.39 (6H, m), ESI-TOF-HRMS *m/z* calcd. for $C_{24}H_{32}NO_9$ $[M+H]^+$ 478.2072, found 478.2088.

Preparation of 17



Synthesis of 17-2

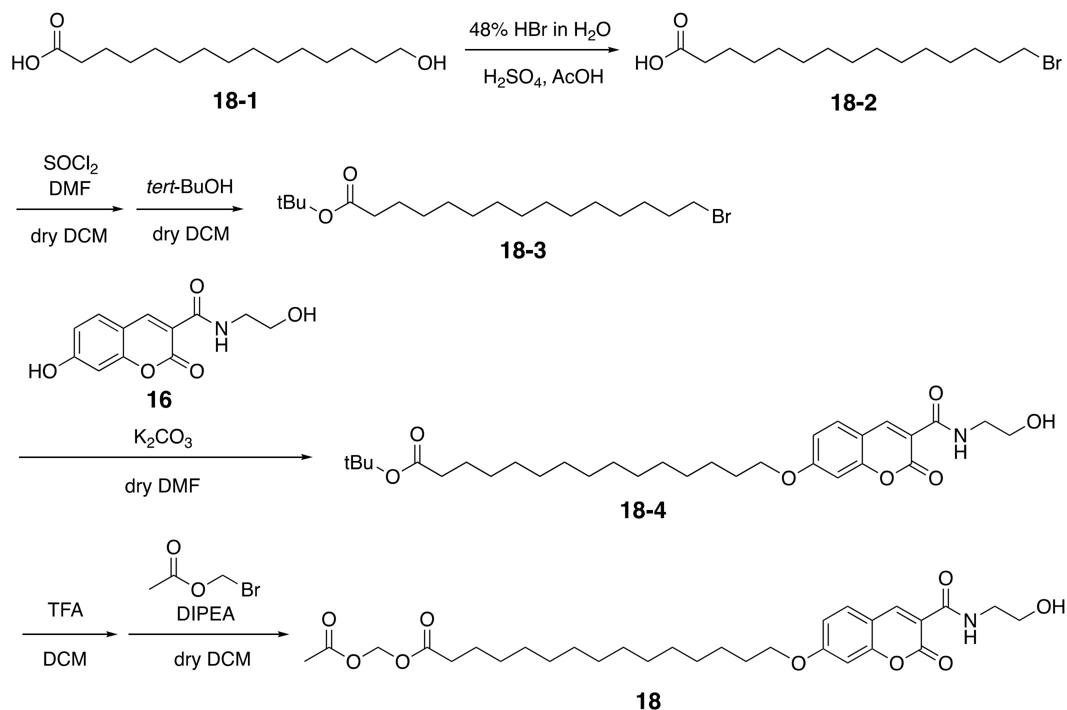
To a solution of **16** (20.0 mg, 80.2 μmol) and K_2CO_3 (44.3 mg, 321 μmol) in dry DMF (1 mL) was added **17-1**[S7] (38.1 mg, 161 μmol) and stirred at 50 °C for 3h. After removal of the solvent by evaporation under reduced pressure, the residue was diluted with chloroform and washed with brine. The crude product was purified by column chromatography on SiO_2 (chloroform) to give **17-2** (100 mg, 75%) as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 9.11 (1H, t, *J* = 6.4 Hz), 8.84 (1H, s), 7.57 (1H, d, *J* = 8.8 Hz), 6.93 (1H, dd, *J* = 2.4, 8.8 Hz), 6.84 (1H, d, *J* = 2.0 Hz), 4.07 (2H, t, *J* = 6.4 Hz), 3.83-3.85 (2H, m), 3.62-3.66 (2H, m), 2.31 (2H, t, *J* = 7.2

Hz), 1.86-1.89 (2H, m), 1.75-1.84 (2H, m), 1.45 (9H, s), ESI-TOF-MS *m/z* calcd. for $C_{21}H_{28}NO_7$ $[M+H]^+$ 406.1866 found, 406.1861

Synthesis of 17

To a solution of **17-2** (10.0 mg, 30.7 μmol) in dichloromethane (2 mL) was added TFA (0.5 mL) and the mixture was stirred at room temperature for 2h. After removal of the solvent by evaporation, the residue was diluted with dry DMF (1 mL). To the solution were added DIPEA (53.4 μL, 307 μmol) and bromomethyl acetate (8.5 μL, 90.2 μmol), and the mixture was stirred at 50 °C for 14h. After removal of the solvent by evaporation, the mixture was diluted with chloroform and washed with brine. After removal of the reaction by evaporation under reduced presser, the residue was purified by column chromatography on SiO_2 (chloroform : methanol = 100 : 1) to give **17** (1.9 mg, 14%) as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 9.11 (1H, t, J = 6.4 Hz), 8.84 (1H, s), 7.58 (1H, d, J = 8.4 Hz), 6.93 (1H, dd, J = 2.4, 8.8 Hz), 6.85 (1H, d, J = 2.0 Hz), 5.75 (2H,s), 4.08 (2H, t, J = 6.4 Hz), 3.81-3.84 (2H, m), 3.62-3.69 (2H, m), 2.48 (2H, t, J = 6.4 Hz), 2.12 (3H, s), 1.81-1.92 (2H, m), 1.75-1.84 (2H, m), ESI-TOF-MS *m/z* calcd. for $C_{20}H_{23}NO_9Na$ $[M+Na]^+$ 444.1271 found, 444.1275

Preparation of 18



Synthesis of 18-2

To a solution of **18-1** (300 mg, 1.16 mmol) in AcOH (5 mL) were added conc. H_2SO_4 (100 µL, 1.86 mmol) and 48% HBr in H_2O (270 µL, 2.32 mmol) and the mixture was stirred at 110 °C for 4h. After quenched with sat. $NaHCO_3$ aq., the crude product was extracted with chloroform. After removal of the reaction by evaporation, the residue was purified by column chromatography on SiO_2 (hexane : ethyl acetate : HCOOH = 60 : 10 : 1) to give **18-2** (258 mg, 69%) as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.41 (2H, t, *J* = 6.8 Hz), 2.35 (2H, t, *J* = 7.6 Hz), 1.82-1.89 (2H, m), 1.60-1.68 (2H, m), 1.43 (2H, t, *J* = 7.4 Hz), 1.26-1.30 (18H, m), ESI-TOF-MS *m/z* calcd. for $C_{15}H_{29}BrO_2Na$ $[M+Na]^+$ 343.1249, found 343.1262

Synthesis of 18-3

To a solution of **18-2** (150 mg, 469 µmol) in dry DCM (2.5 mL) were added thionyl chloride (41.0 µL, 563 µmol) and 2 drops of dry DMF and the mixture was stirred for 1h at room temperature. After removal of the solvent by evaporation under reduced pressure, the residue was dissolved in DCM (2.5 mL) and added *tert*-BuOH (268 µL, 2.81 mmol). The mixture was stirred for 1h at room temperature and quenched by water. The crude product was extracted with ethyl acetate and the combined organic layer was washed with sat. $NaHCO_3$ aq. and brine. After removal of the solvent by evaporation under reduced pressure, the crude product was purified by column chromatography on SiO_2 (hexane : ethyl acetate = 1 : 1) to give **18-3** (90.4 mg, 52%) as colorless oil. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.41 (2H, t, *J* = 7.0 Hz), 2.20 (2H, t, *J* = 7.4 Hz), 1.84-1.87 (2H, m), 1.50-1.54 (2H, m), 1.44-1.46 (11H, m), 1.26-1.28 (18H, m), ESI-TOF-MS *m/z* calcd. for $C_{19}H_{37}BrO_2Na$ $[M+Na]^+$ 399.1875, found 399.1884

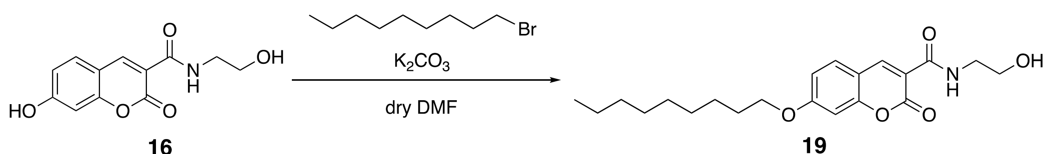
Synthesis of 18-4

To a solution of **16** (50.0 mg, 200 µmol) and K_2CO_3 (83.0 mg, 600 µmol) in 3 mL of dry DMF was added **18-3** (90.4 mg, 240 µmol) and stirred at 80 °C overnight. After removal of the solvent by evaporation under reduced pressure, the residue was diluted with chloroform and washed with brine. The crude product was purified by column chromatography on SiO_2 (hexane : ethyl acetate = 1 : 1) to give **18-4** (40.2 mg, 37%) as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 9.11 (1H, t, *J* = 6.4 Hz), 8.85 (1H, d, *J* = 2.0 Hz), 7.57 (1H, dd, *J* = 2.0, 6.4 Hz), 6.94 (1H, dd, *J* = 1.6, 7.2 Hz), 6.85 (1H, s), 4.06 (2H, t, *J* = 5.2 Hz), 3.81-3.84 (2H, m), 3.62-3.69 (2H, m), 2.20 (2H, t, *J* = 6.0 Hz), 1.81-1.85 (2H, m), 1.44-1.46 (11H, m), 1.23-1.36 (20H, m), ESI-TOF-MS *m/z* calcd. for $C_{31}H_{47}NO_7Na$ $[M+Na]^+$ 568.3250, found 568.3247

Synthesis of 18

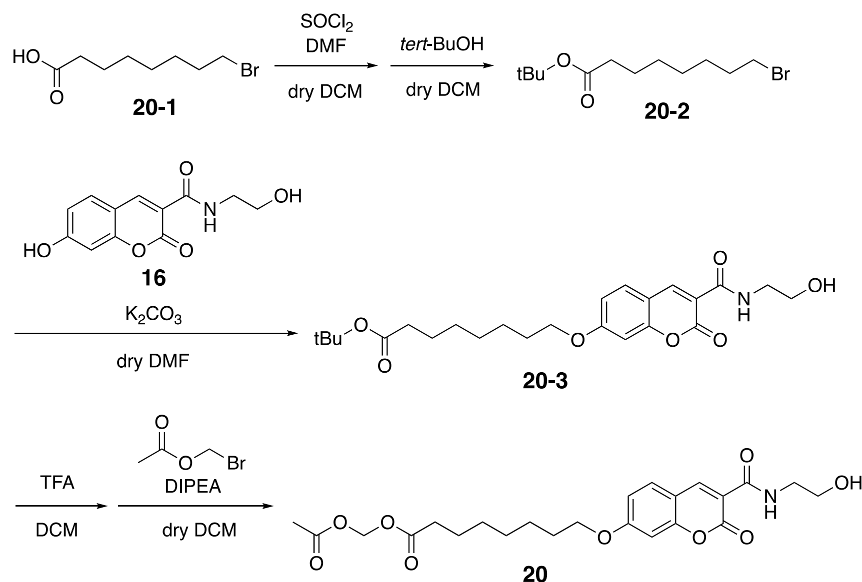
To a solution of **18-4** (40.0 mg, 74.0 μmol) in dichloromethane (1.5 mL) was added TFA (0.5 mL) and the mixture was stirred at room temperature for 1h. After removal of the solvent by evaporation, the residue was diluted with 1 mL of dry DMF. To the solution were added DIPEA (131 μL, 740 μmol) and bromomethyl acetate (24.1 μL, 222 μmol), and the mixture was stirred at 50 °C for 9h. After removal of the solvent by evaporation under reduced presser, the mixture was diluted with chloroform and washed with brine. After removal of the solvent under, the residue was purified by column chromatography on SiO_2 (hexane : ethyl acetate = 1 : 1 to 1 : 10) to give **18** (1.9 mg, 7%) as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 9.11 (1H, t, *J* = 6.8 Hz), 8.84 (1H, s), 7.57 (1H, d, *J* = 6.8 Hz), 6.93(1H, d, *J* = 7.2 Hz), 6.85 (1H, s), 5.74 (2H, s), 4.06 (2H, t, *J* = 5.2 Hz), 3.82-3.84 (2H, m), 3.62-3.66 (2H, m), 2.36 (2H, t, *J* = 6.0 Hz), 2.11 (3H, s), 1.77-1.86 (2H, m), 1.63-1.67 (2H, m), 1.45-1.47 (2H, m), 1.26-1.36 (18H, m), ESI-TOF-HRMS *m/z* calcd. for $C_{30}H_{43}NO_9Na$ $[M+Na]^+$ 584.2836, found 584.2842.

Preparation of 19



To a solution of **16** (15.0 mg, 60.2 μmol) and K_2CO_3 (25.0 mg, 181 μmol) in 1 mL of dry DMF was added 1-bromononane (11.4 μL, 60.2 μmol), and the mixture was stirred overnight at 50 °C. After evaporation of the solvent under reduced pressure, the crude product was diluted with Ethyl acetate and washed with brine. After removal of the solvent by evaporation under reduced presser, the residue was purified by column chromatography on SiO_2 (chlroform : methanol = 20:1) to give **19** (8.5 mg, 38%) as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 9.10 (1H, t, *J* = 4.0 Hz), 8.83 (1H, s), 7.55 (1H, d, *J* = 7.2 Hz), 6.91 (1H, dd, *J* = 1.6, 7.2), 6.83 (1H, d, *J* = 1.6 Hz), 4.04 (2H, t, *J* = 5.6 Hz), 3.82 (2H, t, *J* = 4.0 Hz), 3.61-3.64 (2H, m), 1.80-1.83 (2H, m), 1.43-1.48 (2H, m), 1.24-1.36 (10H, m), 0.87 (3H, t, *J* = 5.6 Hz). ESI-TOF-HRMS *m/z* calcd. for $C_{21}H_{29}O_5NNa$ $[M+Na]^+$ 398.1943, found 398.1941

Preparation of 20



Synthesis of 20-2

To a solution of **20-1** (152 mg, 1.04 mmol) in dry DCM (2 mL) were added thionyl chloride (90.0 μL, 1.25 mmol) and 2 drops of dry DMF and the mixture was stirred for 90 min at room temperature. After removal of the solvent by evaporation under reduced pressure, the residue was dissolved in DCM (2.5 mL) and added *tert*-BuOH (296 μL, 3.12 mmol). The mixture was stirred for 1h at room temperature and quenched by water. The crude product was extracted with ethyl acetate and the combined organic layer was washed with sat. $NaHCO_3$ aq. and brine to give **20-2** (199 mg, 68%) as colorless oil. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.39 (2H, t, *J* = 6.8 Hz), 2.19 (2H, t, *J* = 8.4 Hz), 1.74-1.88 (2H, m), 1.54-1.58 (2H, m), 1.44 (9H, s), 1.25-1.36 (6H, m), ESI-TOF-MS *m/z* calcd. for $C_{12}H_{23}BrO_2Na$ $[M+Na]^+$ 301.0779, found 301.0803

Synthesis of 20-3

To a solution of **16** (75.0 mg, 300 μmol) and K_2CO_3 (124 mg, 900 μmol) in dry DMF (4 mL) was added **19-2** (100 mg, 360 μmol) and stirred overnight at 50 °C. After removal of the solvent by evaporation under reduced pressure, the residue was diluted with ethyl acetate and washed with brine. The crude product was purified by column chromatography on SiO_2 (chloroform : methanol = 30 : 1) to give **20-3** (100 mg, 75%) as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 9.10 (1H, s), 8.84 (1H, s), 7.57 (1H, d, *J* = 8.4 Hz), 6.91-6.94 (1H, m), 6.85 (1H, d, *J* = 2.0 Hz), 4.05 (2H, t, *J* = 7.6 Hz), 3.84 (2H, t, *J* = 5.2 Hz), 3.63-3.66 (2H, m), 2.17-2.21 (2H, m), 1.62-1.66 (2H, m), 1.44 (9H, s), 1.25-1.39 (6H, m), ESI-TOF-MS *m/z* calcd. for $C_{24}H_{33}NO_7Na$ $[M+Na]^+$ 470.2155, found 470.2172

Synthesis of 20

To a solution of **20-3** (39.0 mg, 87.0 μmol) in DCM (1.5 mL) was added TFA (0.5 mL) and the mixture was stirred at room temperature for 1h. After removal of the solvent by evaporation, the residue was diluted with dry DMF (2 mL). To the solution were added DIPEA (154 μL, 870 μmol) and bromomethyl acetate (28.0 μL, 261 μmol), and the mixture was stirred at 50 °C for 5h. After removal of the solvent by evaporation under reduced presser, the mixture was diluted with chloroform and washed with brine. After removal of the reaction by evaporation, the residue was purified by HPLC (YMC-Triart C18, 250×10mmI.D., flow rate; 3.0 mL/min, detection UV (220 nm), mobile phase gradient : CH_3CN (0.1% TFA) / H_2O (0.1% TFA) = 5/95 (0 min) → 55/45 (10 min) → 85/15 (50 min)) to give **20** (2.9 mg, 13%) as a white solid. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 9.13 (1H, t, *J* = 6.4 Hz), 8.84 (1H, s), 7.57 (1H, d, *J* = 8.4 Hz), 6.91-6.94 (1H, m), 6.85 (1H, d, *J* = 2.0 Hz), 5.74 (2H, s), 4.05 (1H, t, *J* = 6.4 Hz), 3.84 (2H, t, *J* = 5.0 Hz), 3.62-3.65 (2H, m), 2.37 (2H, t, *J* = 7.4 Hz), 2.11 (3H, s), 1.79-1.85 (2H, m), 1.67 (2H, t, *J* = 7.0 Hz), 1.48-1.50 (2H, m), 1.38-1.39 (4H, m), ESI-TOF-HRMS *m/z* calcd. for $C_{23}H_{29}NO_9Na$ $[M+Na]^+$ 486.1740, found 486.1720.

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