

Supporting Information for

Protein Knockdown Using Methyl Bestatin-ligand Hybrid Molecules:
Design and Synthesis of Inducers of Ubiquitination-mediated Degradation of
Cellular Retinoic Acid-Binding Proteins

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Experimental Section

General Procedures for Chemical Experiments. Proton nuclear magnetic resonance spectra (^1H NMR) and carbon nuclear magnetic resonance spectra (^{13}C NMR) were recorded on a JEOL JNMGX500 (500 MHz) spectrometer in the indicated solvent. Chemical shifts (δ) are reported in parts per million relative to the internal standard tetramethylsilane. High-resolution mass spectra (HRMS) and fast atom bombardment (FAB) mass spectra were recorded on a JEOL JMA-HX110 mass spectrometer. Bestatin (**1**) was synthesized by Nippon Kayaku Co. Ltd. (Tokyo, Japan). The other chemical reagents and solvents were purchased from Aldrich, Merck, Tokyo Kasei Kogyo, Wako Pure Chemical Industries, and Kanto Kagaku and used without purification. Flash column chromatography was performed using silica gel 60 (particle size 0.060-0.210 mm) supplied by Kanto Kagaku.

(2*E*,4*E*,6*E*,8*E*)-2-Cyanoethyl 9-((*E*)-3-(2-hydroxy-2-oxoethoxyimino)-2,6,6-trimethylcyclohex-1-enyl)-3,7-dimethylnona-2,4,6,8-tetraenoate (5**).**

Step 1; Preparation of (2*E*,4*E*,6*E*,8*E*)-2-cyanoethyl 3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoate. A mixture of ATRA (**3**) (679 mg, 2.19 mmol), 2-cyanoethanol (450 mg, 6.33 mmol), EDCI (627 mg, 3.27 mmol), DMAP (473 mg, 3.87 mmol) in CH_2Cl_2 (4 mL) was stirred at room temperature for 18 hours. Then, the reaction mixture was quenched with water, and extracted with AcOEt. The organic layer was washed with brine, and dried over MgSO_4 . Filtration, evaporation of the solvent *in vacuo* and purification by flash column chromatography (AcOEt/*n*-hexane = 1/6) gave 693 mg (90%) of (2*E*,4*E*,6*E*,8*E*)-2-cyanoethyl 3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-

tetraenoate as a yellow oil; ^1H NMR (CDCl_3 , 500 MHz, δ ; ppm): 7.04 (1H, dd, $J = 14.9, 11.3$ Hz), 6.31 (1H, s), 6.28 (1H, s), 6.14 (2H, m), 5.79 (1H, s), 4.32 (2H, t, $J = 6.1$ Hz), 2.73 (2H, t, $J = 6.0$ Hz), 2.37 (3H, s), 2.02 (5H, m), 1.72 (3H, s), 1.62 (2H, m), 1.42 (2H, m), 1.03 (6H, s); MS (FAB) m/z : 353 (M^+).

Step 2; Preparation of (2E,4E,6E,8E)-2-cyanoethyl 3,7-dimethyl-9-(2,6,6-trimethyl-3-oxocyclohex-1-enyl)nona-2,4,6,8-tetraenoate. A mixture of (2E,4E,6E,8E)-2-cyanoethyl 3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoate (56.0 mg 0.158 mmol) and MnO_2 (1300 mg, 14.95 mmol) in CH_2Cl_2 (5 mL) was stirred at room temperature for 22 hours. Then, MnO_2 was removed by filtration and the filtrate was concentrated *in vacuo*. The residue was purified by flash column chromatography ($\text{AcOEt}/n\text{-hexane} = 1/4$ to $1/1$) gave 21.1 mg (36%) of (2E,4E,6E,8E)-2-cyanoethyl 3,7-dimethyl-9-(2,6,6-trimethyl-3-oxocyclohex-1-enyl)nona-2,4,6,8-tetraenoate as a yellow oil; ^1H NMR (CDCl_3 , 500 MHz, δ ; ppm): 7.03 (1H, dd, $J = 14.9, 11.3$ Hz), 6.37 (1H, d, $J = 15.2$ Hz), 6.34 (1H, s), 6.27 (1H, d, $J = 11.0$ Hz), 5.84 (1H, s), 4.33 (2H, t, d, $J = 6.4$ Hz), 2.74 (2H, t, $J = 6.4$ Hz), 2.52 (2H, t, $J = 6.7$ Hz), 2.37 (3H, s), 2.04 (3H, s), 1.86 (5H, m), 1.19 (6H, s); MS (FAB) m/z : 368 (MH^+).

Step 3; Preparation of (2E,4E,6E,8E)-2-cyanoethyl 9-((E)-3-(2-hydroxy-2-oxoethoxyimino)-2,6,6-trimethylcyclohex-1-enyl)-3,7-dimethylnona-2,4,6,8-tetraenoate (5). A mixture of (2E,4E,6E,8E)-2-cyanoethyl 3,7-dimethyl-9-(2,6,6-trimethyl-3-oxocyclohex-1-enyl)nona-2,4,6,8-tetraenoate (180 mg 0.490 mmol) and *O*-(carboxymethyl)hydroxylamine (157 mg, 1.44 mmol) in pyridine (8 mL) was stirred at room temperature for 17 hours. The reaction mixture was poured into 10% aqueous citric acid and extracted with AcOEt. The organic layer was washed with brine and dried over MgSO_4 . Filtration,

evaporation of the solvent *in vacuo* and purification by flash column chromatography (AcOEt/*n*-hexane = 1/1) gave 215 mg (100%) of **5** as a yellow amorphous solid; ¹H NMR (CDCl₃, 500 MHz, δ; ppm): 7.04 (1H, dd, *J* = 14.9, 11.3 Hz), 7.37–6.20 (4H, m), 5.83 (1H, s), 4.68 (2H, s), 4.33 (2H, t, *J* = 6.4 Hz), 2.74 (2H, t, *J* = 6.4 Hz), 2.70 (2H, t, *J* = 6.7 Hz) 2.37 (3H, s), 2.03 (3H, s), 1.88 (3H, s), 1.63 (2H, t, *J* = 6.7 Hz), 1.10 (6H, s); MS (FAB) *m/z*: 441 (MH⁺).

(*S*)-2-[(2*S*,3*R*)-3-[(9*H*-Fluoren-9-yl)methoxy]carbonylamino-2-hydroxy-4-phenylbutanamido]-4-methylpentanoic acid (6**).** A solution of FmocCl (1156 mg, 4.47 mmol) in THF (40 mL) was added to a solution of bestatin (**1**) (1171 mg, 4.47 mmol) and K₂CO₃ (1182 mg, 8.55 mmol) in H₂O/THF (20 mL/40 mL) in a dropwise fashion with cooling in an ice-bath. The reaction mixture was stirred room temperature for 24 hours, then acidified with 2 M HCl, and the organic solvent was removed by concentration *in vacuo*. The residue was extracted with AcOEt. The organic layer was washed with brine, and dried over MgSO₄. Filtration, evaporation of the solvent *in vacuo* and purification of the residue by flash column chromatography (AcOEt/*n*-hexane = 1/2 to AcOEt only) gave 1950 mg (97%) of **6** as a colorless solid; ¹H NMR (CDCl₃, 500 MHz, δ; ppm): 7.75 (2H, d, *J* = 7.5 Hz), 7.48 (2H, t, *J* = 7.5 Hz), 7.40 (2H, t, *J* = 8.0 Hz), 7.31–7.20 (7H, m), 5.37 (1H, d, *J* = 8.0 Hz), 4.55 (1H, m), 4.34 (1H, m), 4.21 (2H, dd, *J* = 7.4, 3.0 Hz), 4.14 (1H, m), 3.06 (1H, s), 3.04 (1H, s), 1.70 (1H, m), 1.59 (2H, m), 0.89 (3H, d, *J* = 6.0 Hz), 0.84 (3H, d, *J* = 6.5 Hz); MS (FAB) *m/z*: 531 (MH⁺).

(*S*)-2-[2-(2-Aminoethoxy)ethoxy]ethyl 2-[(2*S*,3*R*)-3-[(9*H*-fluoren-9-yl)methoxy]carbonyl]-2-hydroxy-4-phenylbutanamido)-4-methylpentanoate hydrochloride (9b**•HCl).**

Step 1; Preparation of (S)-2-[2-[2-(*tert*-butoxycarbonylamino)ethoxy]ethoxy]ethyl 2-((2*S*,3*R*)-3-[[*(9H*-fluoren-9-yl)methoxy]carbonylamino]-2-hydroxy-4-phenylbutanamido)-4-methylpentanoate (8b**).** EDCI (26.7 mg, 0.139 mmol) was added to a solution of **6** (48.1 mg, 0.0907 mmol), **7b**¹ (224 mg, 0.899 mmol), HOBt•H₂O (28.6 mg, 0.187 mmol) and *i*-Pr₂NEt (22.0 μL, 0.117 mmol) in CH₂Cl₂ (4 mL) with cooling in an ice-bath. The resulting mixture was stirred at room temperature for 17 hours. Then, the reaction mixture was quenched with water, and extracted with AcOEt. The organic layer was washed with brine, and dried over MgSO₄. Filtration, evaporation of the solvent *in vacuo* and purification by flash column chromatography (AcOEt/*n*-hexane = 4/1 to AcOEt only) gave 25.0 mg (36%) of **8b** as a colorless oil; ¹H NMR (CDCl₃, 500 MHz, δ; ppm): 7.75 (2H, d, *J* = 7.3 Hz), 7.51 (2H, t, *J* = 7.3 Hz), 7.40 (2H, t, *J* = 7.6 Hz), 7.31–7.14 (7H, m), 5.50 (1H, m), 5.08 (1H, m), 4.61 (1H, m), 4.40–4.15 (5H, m), 3.64 (2H, t, *J* = 4.3 Hz), 3.57 (4H, s), 3.51 (2H, t, *J* = 4.8 Hz), 3.28 (2H, brd, *J* = 4.3 Hz), 3.05 (2H, m), 1.62 (3H, m), 1.44 (9H, s), 0.86 (6H, dd, *J* = 6.2, 5.4 Hz); MS (FAB) *m/z*: 784 (MNa⁺), 762 (MH⁺), 622 (MH⁺-Boc).

Step 2; Preparation of (S)-2-[2-(2-aminoethoxy)ethoxy]ethyl 2-((2*S*,3*R*)-3-[[*(9H*-fluoren-9-yl)methoxy]carbonylamino]-2-hydroxy-4-phenylbutanamido)-4-methylpentanoate hydrochloride (9b•HCl**).** 4 M HCl in 1,4-dioxane (120 μL, 0.480 mmol) was added to **8b** (54.1 mg, 0.0710 mmol) with cooling in an ice-bath and the resulting mixture was stirred at room temperature for 1 hour. The reaction mixture was concentrated *in vacuo* to give crude **9b•HCl** that was used in next step without further purification; MS (FAB) *m/z*: 662 (MH⁺-HCl).

(2E,4E,6E,8E)-9-[(E)-3-(2-{2-[2-(2-{(S)-2-[(2S,3R)-3-Amino-2-hydroxy-4-phenylbutanamido]-4-methylpentanoyloxy}ethoxy)ethoxy]ethylamino}-2-oxoethoxyimino)-2,6,6-trimethylcyclohex-1-enyl]-3,7-dimethylnona-2,4,6,8-tetraenoic acid (4b).

Step 1; Preparation of (2E,4E,6E,8E)-2-cyanoethyl 9-((E)-3-{2-[2-(2-{(S)-2-((2S,3R)-3-{[(9H-fluoren-9-yl)methoxy]carbonylamino}-2-hydroxy-4-phenylbutanamido)-4-methylpentanoyloxy]ethoxy}ethoxy)ethylamino]-2-oxoethoxyimino}-2,6,6-trimethylcyclohex-1-enyl)-3,7-dimethylnona-2,4,6,8-tetraenoate (10b). EDCI (22.0 mg, 0.115 mmol) was added to a solution of **5** (31.0 mg, 0.064 mmol), crude **9b•HCl**, HOBt•H₂O (21.1 mg, 0.137 mmol) and Et₃N (14.0 μL, 0.101 mmol) in CH₂Cl₂ (4 mL) with cooling in an ice-bath. The resulting mixture was stirred at room temperature for 16 hours. Then, the reaction mixture was quenched with water, and extracted with AcOEt. The organic layer was washed with brine, and dried over MgSO₄. Filtration, evaporation of the solvent *in vacuo* and purification of the residue by flash column chromatography (CHCl₃/MeOH = 20/1) gave crude **10b** that was used in next step without further purification; MS (FAB) *m/z*: 1084 (M⁺).

Step 2; Preparation of (2E,4E,6E,8E)-9-[(E)-3-(2-{2-[2-(2-{(S)-2-[(2S,3R)-3-amino-2-hydroxy-4-phenylbutanamido]-4-methylpentanoyloxy}ethoxy]ethoxy}ethylamino)-2-oxoethoxyimino)-2,6,6-trimethylcyclohex-1-enyl]-3,7-dimethylnona-2,4,6,8-tetraenoic acid (4b). 1 M TBAF in THF (516 μL, 0.516 mmol) was added to a solution of crude **10b** and MeOH (44.0 μL, 1.09 mmol) in THF (815 μL). The reaction mixture was stirred at room temperature for 1 hour, then purified by flash column chromatography (CHCl₃/MeOH = 20/1) to give 33.9 mg of crude **11b** as a yellow oil; MS (FAB) *m/z*:

1097 (MNa⁺), 1075 (MH⁺). DBU (20.0 μ L, 0.134 mmol) was added to a solution of crude **11b** and dodecyl mercaptan (17.0 μ L, 0.0722 mmol) in CH₂Cl₂ (1 mL). The reaction mixture was stirred at room temperature for 1 hour, then purified by flash column chromatography (CHCl₃/MeOH = 9/1) and PTLC (CHCl₃/MeOH/NH₃ aqueous = 9/2/0.2) to give 15.2 mg (36%; 3 steps from **5**) of **4b** as a yellow oil; ¹H NMR (CDCl₃, 500 MHz, δ ; ppm): 7.87 (1H, d, J = 6.7 Hz), 7.30–7.24 (5H, m), 6.97 (1H, dd, J = 15.2, 12.1 Hz), 6.73 (1H, s), 6.38–6.18 (4H, m), 5.81 (1H, s), 4.58 (3H, m), 4.29 (2H, m), 4.13 (1H, m), 3.72–3.50 (13H, m), 3.11 (1H, m), 2.66 (3H, m), 2.33 (3H, s), 2.02 (3H, s), 1.86 (3H, s), 1.67 (5H, m), 1.09 (6H, s), 0.93 (3H, d, J = 6.1 Hz), 0.92 (3H, d, J = 6.0 Hz); ¹³C NMR (CDCl₃, 125 MHz, δ ; ppm): 172.62, 170.50, 170.49, 158.80, 150.27, 139.33, 138.30, 137.60, 136.77, 131.65, 130.33, 129.35, 128.83, 128.80, 126.86, 126.58, 124.81, 73.02, 70.54, 70.43, 70.20, 69.94, 68.94, 64.18, 57.48, 50.84, 40.87, 38.70, 35.95, 34.88, 29.68, 27.61, 24.91, 22.42, 21.73, 20.19, 14.89, 13.84, 12.81; MS (FAB) m/z : 809 (MH⁺); HRMS (FAB) calcd for C₄₄H₆₅N₄O₁₀⁺, 808.470; found 808.475.

(2E,4E,6E,8E)-9-((E)-3-{2-[2-(2-{(S)-2-[(2S,3R)-3-Amino-2-hydroxy-4-phenylbutanamido]-4-methylpentanoyloxy}ethoxy)ethylamino]-2-oxoethoxyimino}-2,6,6-trimethylcyclohex-1-enyl)-3,7-dimethylnona-2,4,6,8-tetraenoic acid (4a). Compound **4a** (18.2 mg, 46% from **5**) was prepared from **5**, **6** and **7a**¹ using the procedure described for **9b** and **4b**; yellow oil; ¹H NMR (CDCl₃, 500 MHz, δ ; ppm): 7.83 (1H, d, J = 6.7 Hz), 7.31–7.23 (5H, m), 6.92 (1H, dd, J = 15.2, 12.1 Hz), 6.73 (1H, s), 6.36–6.17 (4H, m), 5.82 (1H, s), 4.60 (3H, m), 4.27 (2H, m), 4.08 (1H, m), 3.66–3.48 (9H, m), 3.04 (1H, m), 2.66 (3H, m), 2.30 (3H, s), 2.00 (3H, s), 1.87 (3H, s), 1.67 (5H, m), 1.09 (6H, s), 0.93 (3H, d, J = 6.1 Hz),

0.92 (3H, d, $J = 6.0$ Hz); ^{13}C NMR (CDCl_3 , 125 MHz, δ ; ppm): 173.00, 172.59, 170.54, 158.92, 150.44, 139.44, 138.14, 138.01, 136.93, 131.78, 130.54, 129.32, 128.76, 126.75, 126.42, 124.71, 73.00, 70.20, 69.99, 68.78, 64.22, 54.57, 50.78, 40.92, 38.74, 35.92, 34.88, 27.59, 24.93, 22.83, 21.74, 20.22, 14.90, 13.82, 12.79; MS (FAB) m/z : 765 (MH^+); HRMS (FAB) calcd for $\text{C}_{42}\text{H}_{61}\text{N}_4\text{O}_9^+$, 765.444; found 765.442.

(2E,4E,6E,8E)-9-[(E)-3-[2-(2-[2-(2-[(S)-2-[(2S,3R)-3-Amino-2-hydroxy-4-phenylbutanamido]-4-methylpentanoyloxy)ethoxy)ethoxy]ethoxy}ethylamino)-2-oxoethoxyimino]-2,6,6-trimethylcyclohex-1-enyl]-3,7-dimethylnona-2,4,6,8-tetraenoic acid (4c). Compound **4c** (8.01 mg, 25% from **5**) was prepared from **5**, **6** and **7c**^{1,2} using the procedure described for **9b** and **4b**; yellow oil; ^1H NMR (CDCl_3 , 500 MHz, δ ; ppm): 7.92 (1H, d, $J = 6.7$ Hz), 7.32–7.20 (5H, m), 6.97 (1H, dd, $J = 15.2, 12.1$ Hz), 6.80 (1H, s), 6.28 (4H, m), 5.80 (1H, s), 4.57 (2H, s), 4.33 (1H, m), 4.24 (2H, m), 3.72–3.50 (17H, m), 3.17 (2H, m), 2.82 (1H, m), 2.67 (3H, t, $J = 6.7$ Hz), 2.31 (3H, s), 2.02 (3H, s), 1.87 (3H, s), 1.67 (5H, m), 1.09 (6H, s), 0.93 (3H, d, $J = 6.1$ Hz), 0.92 (3H, d, $J = 6.0$ Hz); ^{13}C NMR (CDCl_3 , 125 MHz, δ ; ppm): 172.37, 172.25, 170.62, 170.61, 158.85, 150.22, 139.32, 136.55, 136.59, 133.05, 131.58, 130.61, 129.45, 128.87, 128.85, 127.05, 126.73, 124.87, 73.00, 70.56, 70.41, 70.36, 70.33, 70.04, 70.03, 69.89, 69.02, 64.19, 57.21, 51.13, 40.39, 38.71, 35.95, 34.86, 29.69, 27.62, 24.83, 22.82, 21.72, 20.19, 14.92, 13.88, 12.82; MS (FAB) m/z : 853 (MH^+); HRMS (FAB) calcd for $\text{C}_{48}\text{H}_{69}\text{N}_4\text{O}_{11}^+$, 853.496; found 853.494.

Cell Culture Conditions. Human fibrosarcoma HT-1080 cells, human leukemia, acute T lymphoblastic MOLT-4 cells and human leukemia HL-60 cells were cultured in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS), penicillin and streptomycin mixture at 37 °C in a humidified atmosphere of 5% CO₂ in air. Human neuroblastoma IMR-32 cells were cultured in Eagle's minimal essential medium with non-essential amino acids and 10% FBS at 37 °C in a humidified atmosphere of 5% CO₂ in air. Human embryonic kidney (HEK) 293 cells were cultured in D-MEM medium containing 5% FBS at 37 °C in a humidified atmosphere of 5% CO₂ in air.

FLAG-cIAP1 transfection. The transfection experiments were carried out according to the method reported in ref 3.

Western Blotting. HT1080, FLAG-cIAP1 HT1080, MOLT-4 and IMR-32 (1×10^6) cells were treated for the indicated period with MeBS (**2**) (Nippon Kayaku Co. Ltd.), ATRA (**3**) (Tokyo Kasei Kogyo), MG132 (Peptide Institute Inc.), lactacystin (Toronto Research Chemicals Inc.) and/or synthetic compounds at the indicated concentrations in 10% FBS-supplemented in appropriate cell culture medium, then the cells were collected and extracted with SDS buffer. Protein concentrations of the lysates were determined using a BCA protein assay. Equivalent amounts of protein from each lysate were resolved in 10–20% SDS-polyacrylamide gels and transferred onto PVDF membranes. After blocking with TBS containing 5% skim milk, the transblotted membranes were probed with mouse monoclonal CRABP-I antibody (Adcam) (1:5000 dilution), rabbit polyclonal CRABP-II antibody (Novus Biologicals, Inc.) (1:1000 dilution), affinity-purified goat cIAP1 antibody (R & D systems) (0.5

μg/mL), mouse monoclonal RARα antibody (Perseus Proteomics Inc.) (1:1000 dilution), anti-goat IgG-horseradish peroxidase conjugates (Kirkegaard & Perry Laboratories, Inc.) (1:5000 dilution), anti-mouse IgG-horseradish peroxidase conjugates (Chemicon) (1:2000 dilution), goat anti-rabbit IgG-horseradish peroxidase conjugates (Amersham) (1:2000 dilution), FLAG antibody (SIGMA) (1:500 dilution), β-actin antibody (Santa Cruz Biotechnology, Inc.) (1:2000 dilution) in can-get-signal solution (TOYOBO). After probing, the membrane was washed twice more with TBS-T. The immunoblots were visualized by enhanced chemiluminescence with Immobilon™ Western Chemiluminescent HRP Substrate (Millipore).

Pull down assay. Pull down assays were done with GST•Bind™ Kits (Novagen). Recombinant GST-BIR3 and GST were produced and purified as described previously.³ 1 μM GST-BIR3 or 1 μM GST were added a solution of 10 nM CRABP-II (ATGen Co., Ltd.) and 10 μM synthetic compounds in buffer (50 mM PBS-NaOH, pH 7.4, 150 mM NaCl). The solution and a suspension of 40 μL GST•Bind Resin were added to 0.5 mL microtubes and the mixture was incubated at room temperature for 30 minutes with gentle agitation. After centrifugation and removal of the supernatant, the resin was washed with 20 μL of Wash buffer. The washing was repeated three times. Then, protein bound to the resin was eluted with Elution buffer. The solution was poured into an equal volume of 2 x SDS sample buffer and the sample was analyzed by Western Blotting using anti-GST antibody (Amersham) (1:5000 dilution) and rabbit polyclonal CRABP-II antibody (Novus Biologicals, Inc.).

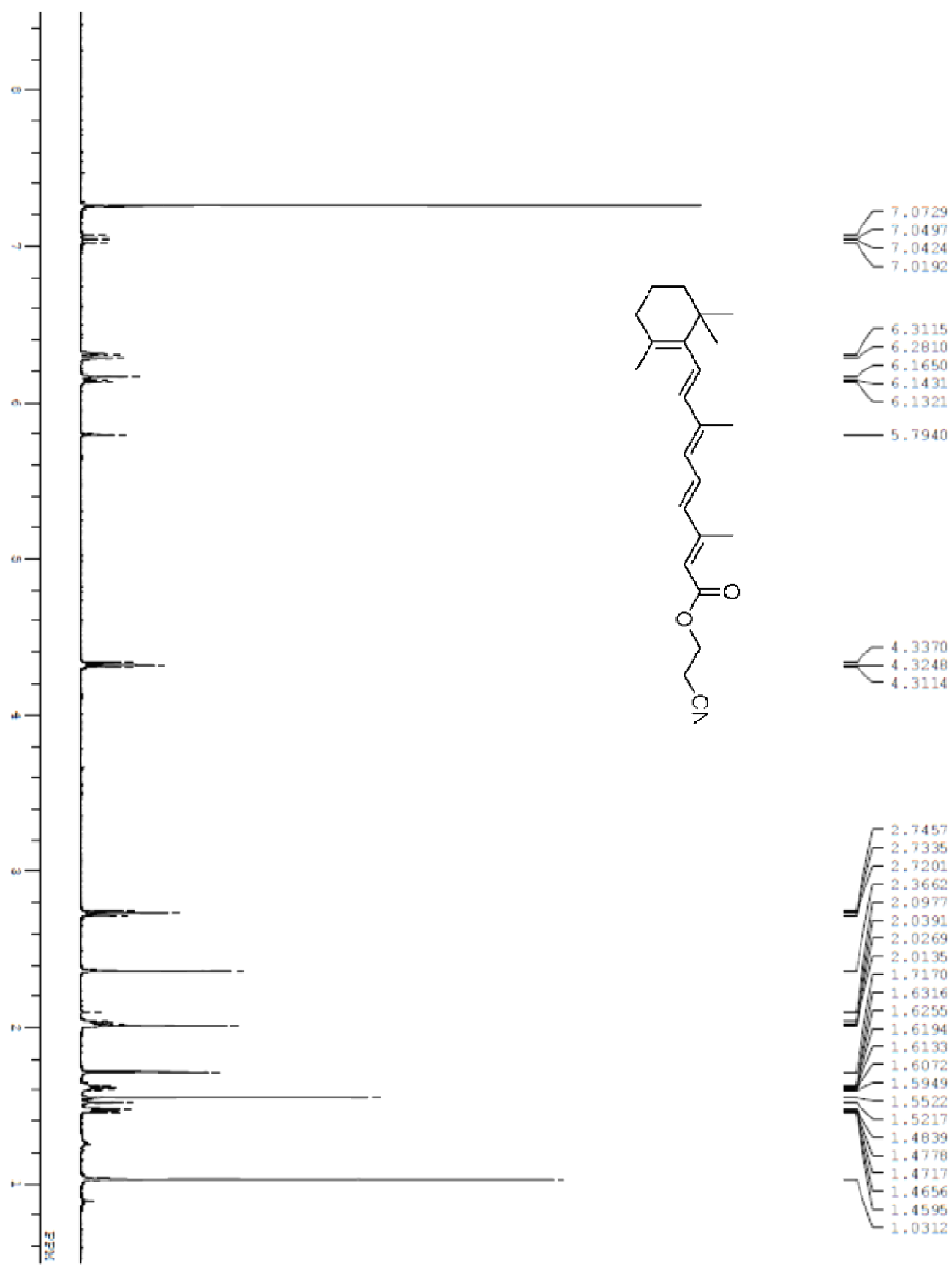
Cell migration assay.⁴ Migration assays were done in 6.5 mm Transwells[®] with 8.0 μm pore polycarbonate membrane inserts (Corning Inc.). IMR-32 cells (1.0×10^6) were seeded in Transwells in triplicate under culture medium containing test compounds, and the same medium was added to the bottom wells. The cells were allowed to migrate for 24 hours. Then, the numbers of cells in the bottom wells were counted.

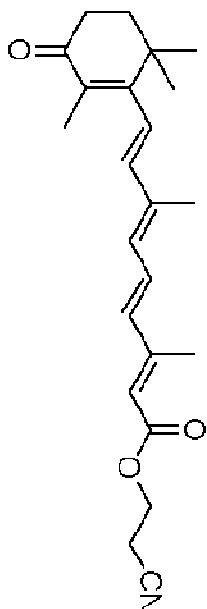
Reporter gene assay. Transfections of CMX-GAL4N-hRAR α , CMX-GAL4N-hRAR β , CMX-GAL4N-hRAR γ and CMX- β -GAL (provided by Prof. Makishima) into HEK 293 cells were performed by the calcium phosphate coprecipitation method. 100 nM ATRA (positive control) and, 0.3 μM , 3 μM and 30 μM **4b** were added 6 h later. After overnight incubation, luciferin was added and luminescence was measured on ARVO[™] SX microplate reader. β -Galactosidase was added and the absorbance was measured on the microplate reader with emission detection set at 405 nm.

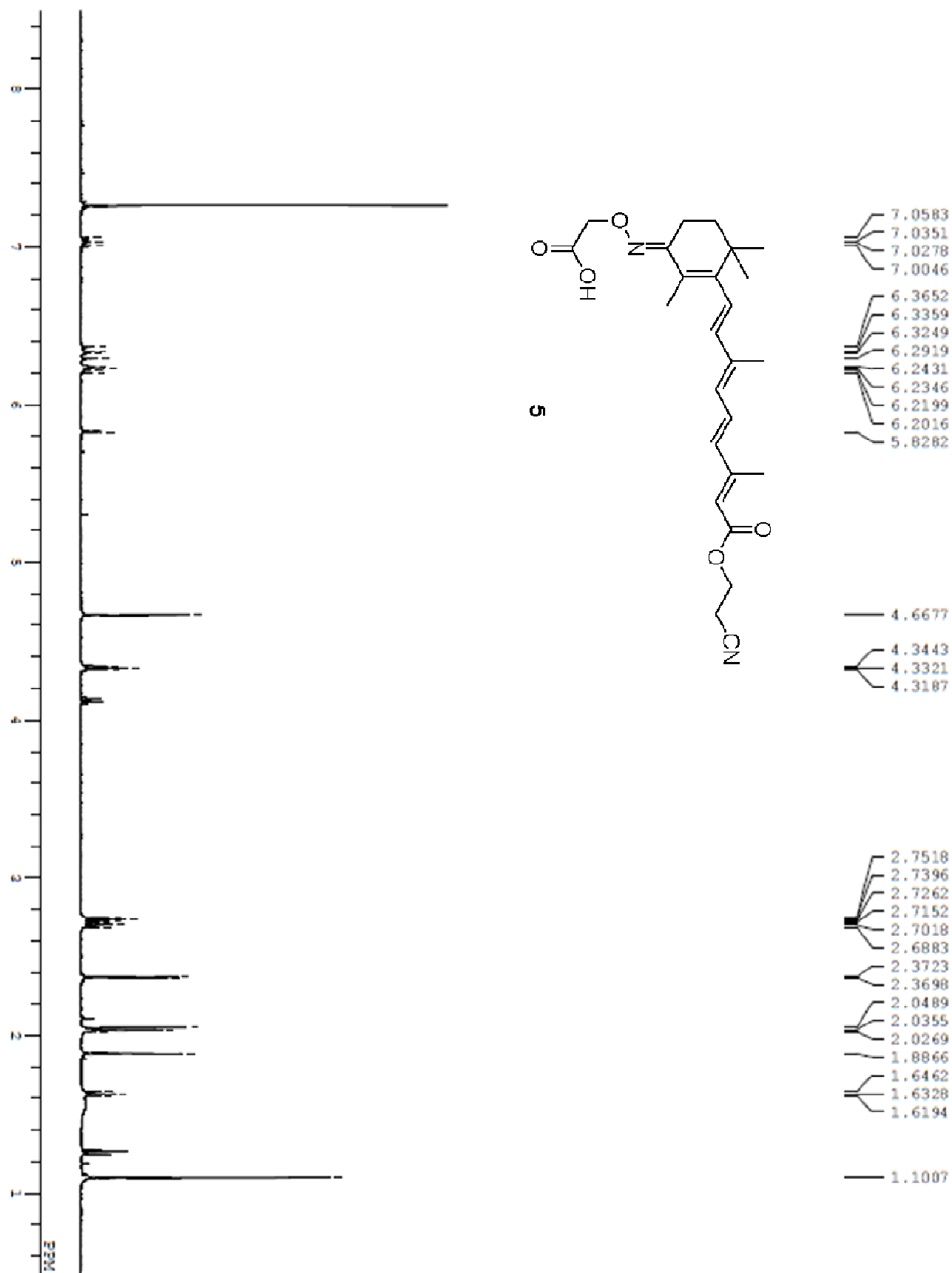
Assay of cell differentiation-inducing activity. HL-60 cells in 1×10^5 cells/ml were incubated in RPMI1640 medium in the presence or absence of ATRA (**3**) or compound **4b** for 3 days. Treated HL-60 cells were harvested via centrifugation and incubated with an equal volume of 0.2% (w/v) nitro blue tetrazolium (NBT) (Wako Pure Chemical Industries) dissolved in PBS, containing 20 nM 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (Wako Pure Chemical Industries) dissolved in EtOH at 37 °C for 30 min. NBT positivity was measured by counting and the results were expressed as the percentage of NBT-positive cells.

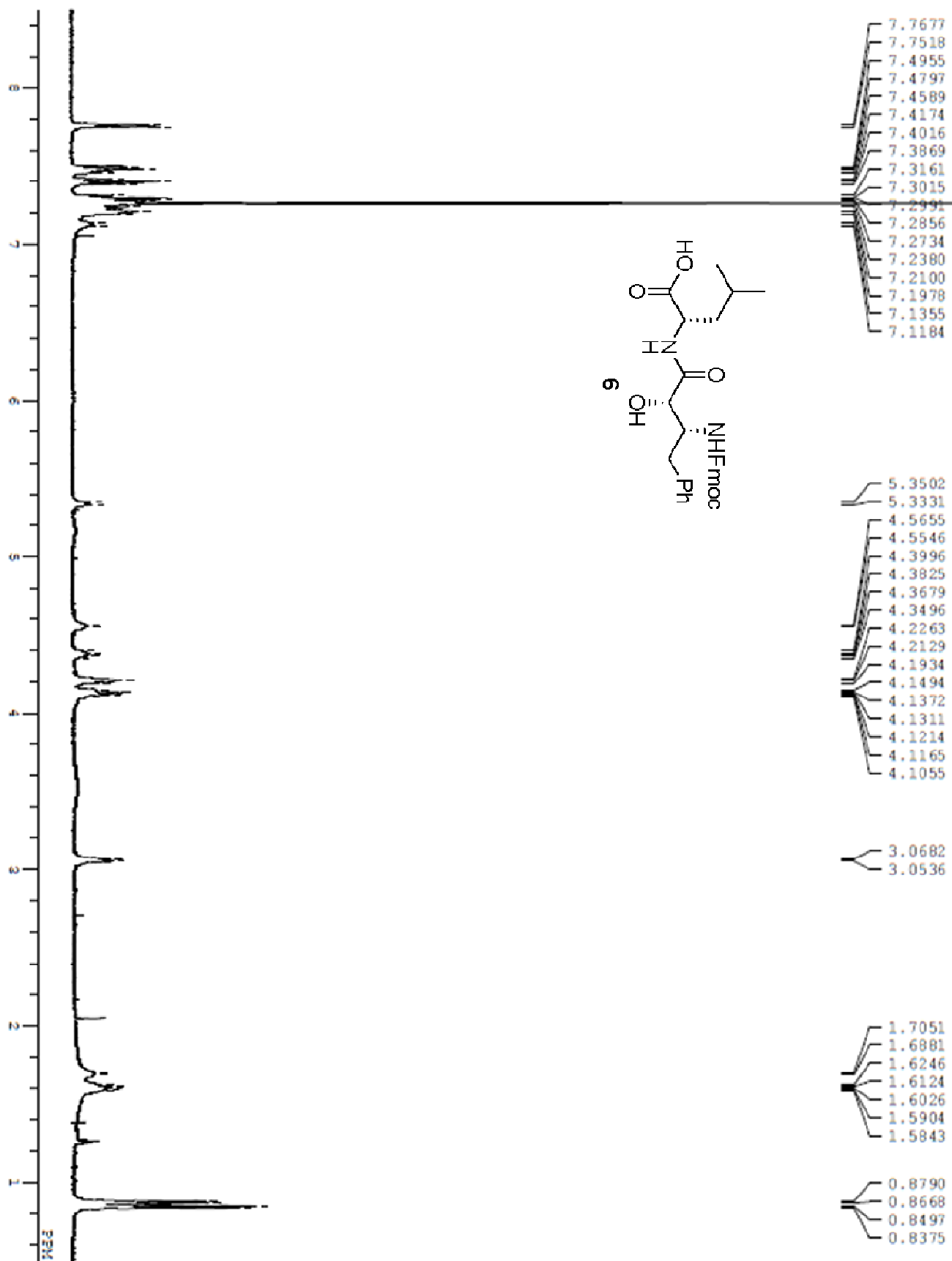
RARs binding assay.⁵ RARs fraction was prepared from HL-60 cells as previously described.⁵

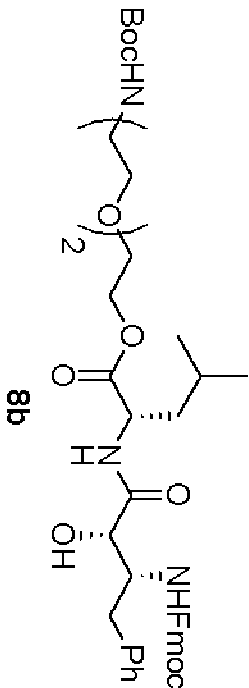
RARs fraction was diluted with a binding assay buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) and incubated with 4 nM of ^3H -Am80, which is potent RARs agonist and possesses very low affinity toward CRABP,^{5a, 6} in the presence of 1000 nM of ATRA (**3**) and 1000 nM of compound **4b** for 8 hours at 4 °C. The incubation mixture was absorbed by suction onto a nitrocellulose membrane that had been soaked in binding assay buffer. The membrane was washed with buffer (20 mM Tris-HCl, pH 8.0, 150 mM KCl) and then with 25% ethanol in distilled water. Radioactivity of the supernatant was measured in scintillation cocktail (AQUASOL[®]-2, Packard BioScience) using a liquid scintillation counter (PACKARD, TRI-CARB 2700TR).



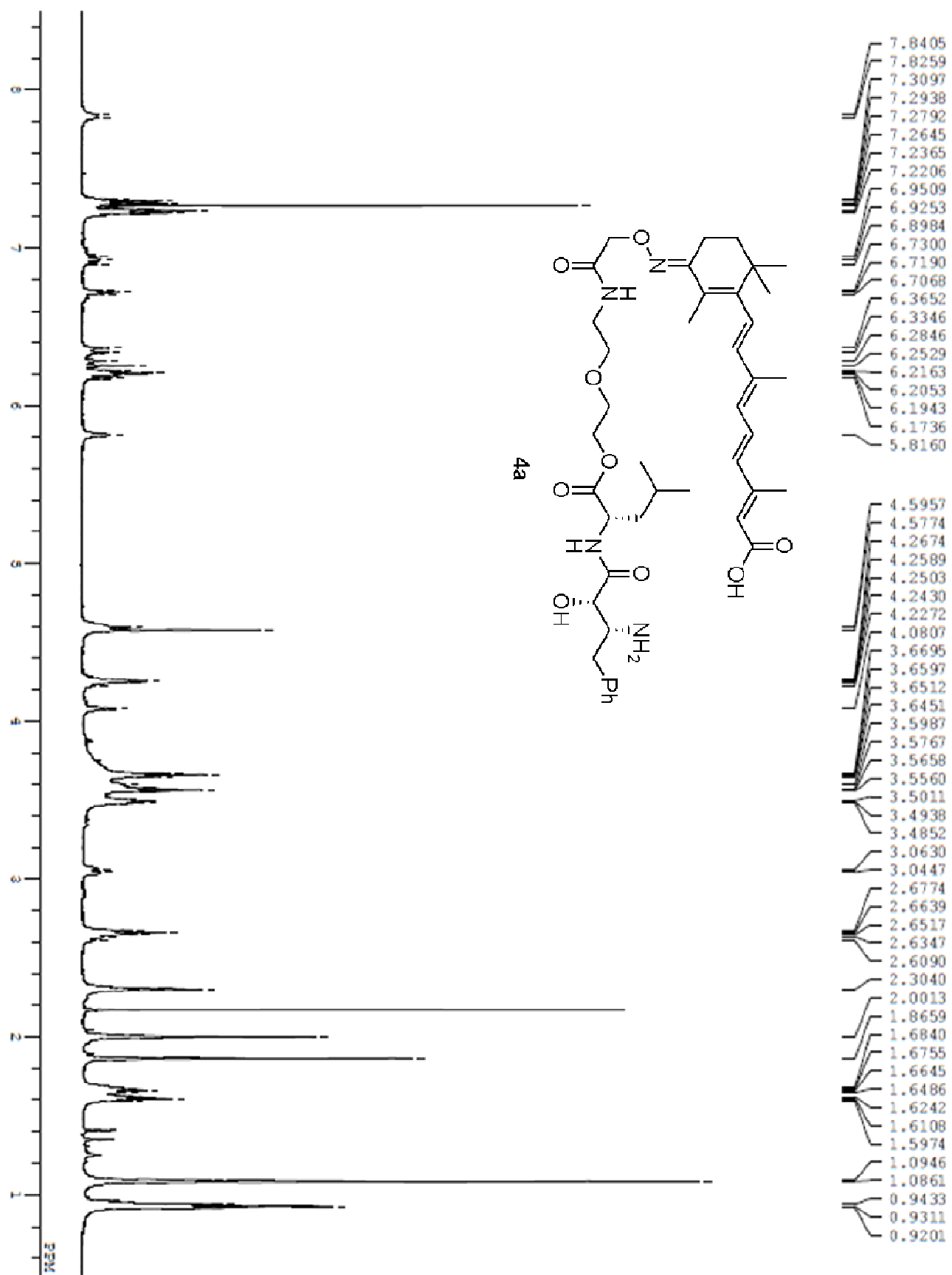


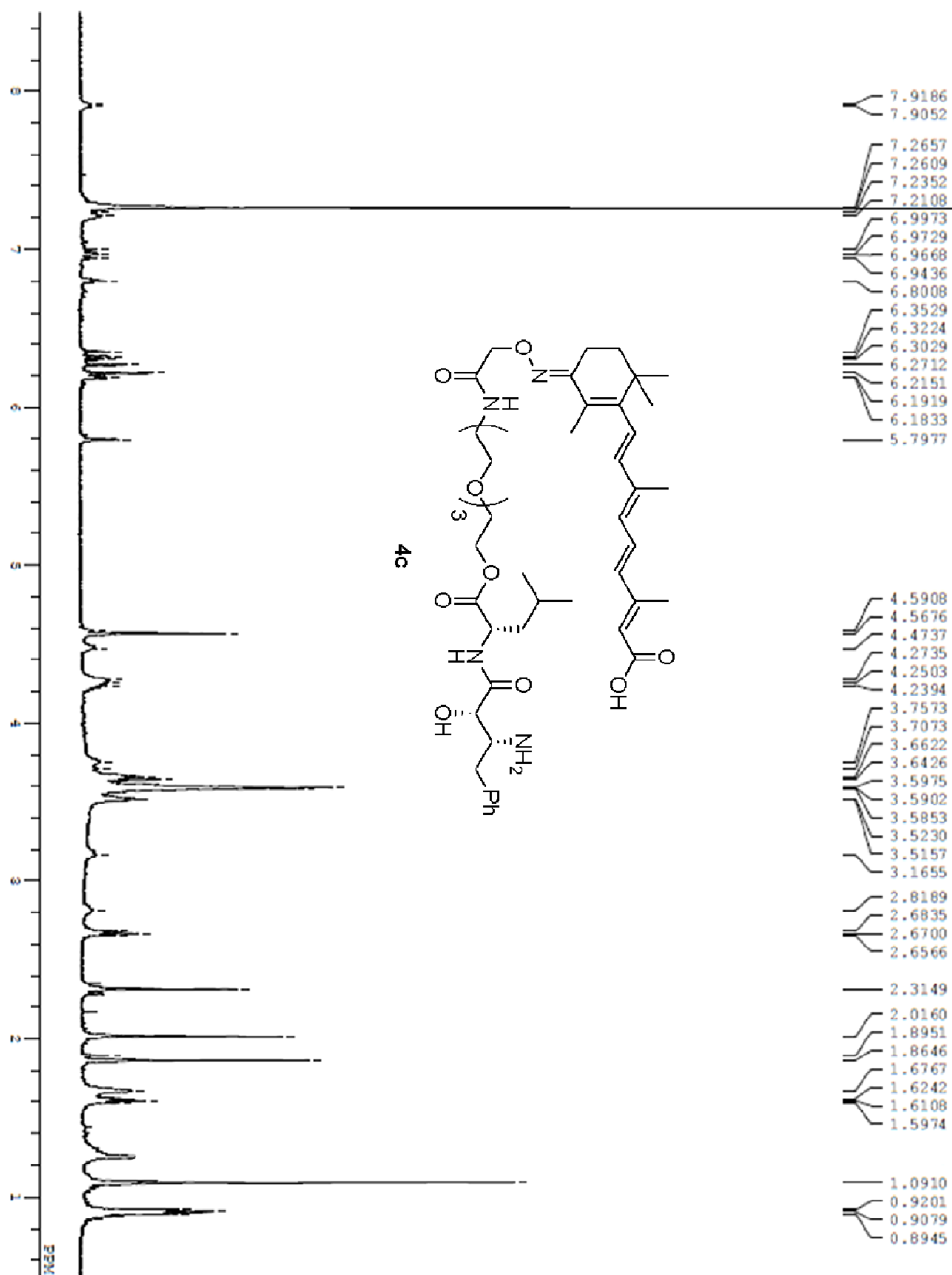












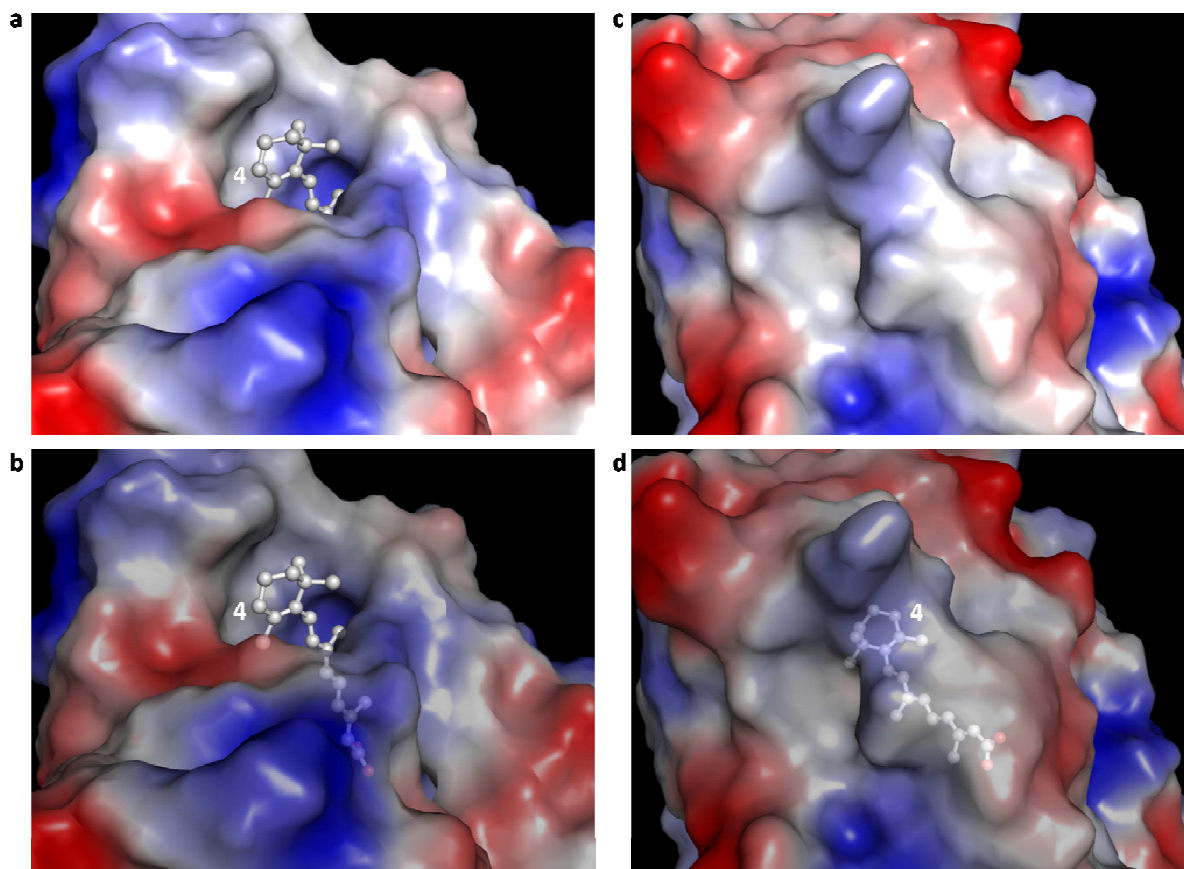


Figure S1. Electronic surface of the ligand-binding domain of CRABP-II (PDB ID; 1CBS) and RAR γ (PDB ID; 2LBD). Acidic domain (red), basic domain (blue), hydrophobic domain (white). ATRA is represented by a ball and stick model. (a) Electronic surface of CRABP-II. (b) Same view as Fig. S1a but the electronic surface is represented as transparent. The C4 position of ATRA (**3**) is outside the protein. (c) Electronic surface of RAR γ . (d) Same view as Fig. S1c but the electronic surface is represented as transparent. ATRA (**3**) is buried in the protein.

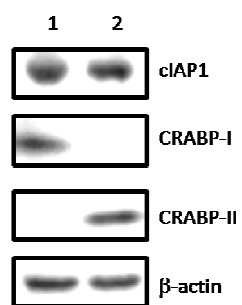


Figure S2. Western blot detection of CRABP-I, CRABP-II and cIAP1 levels. Lane 1, MOLT-4 cells.

Lane 2, HT1080 cells.

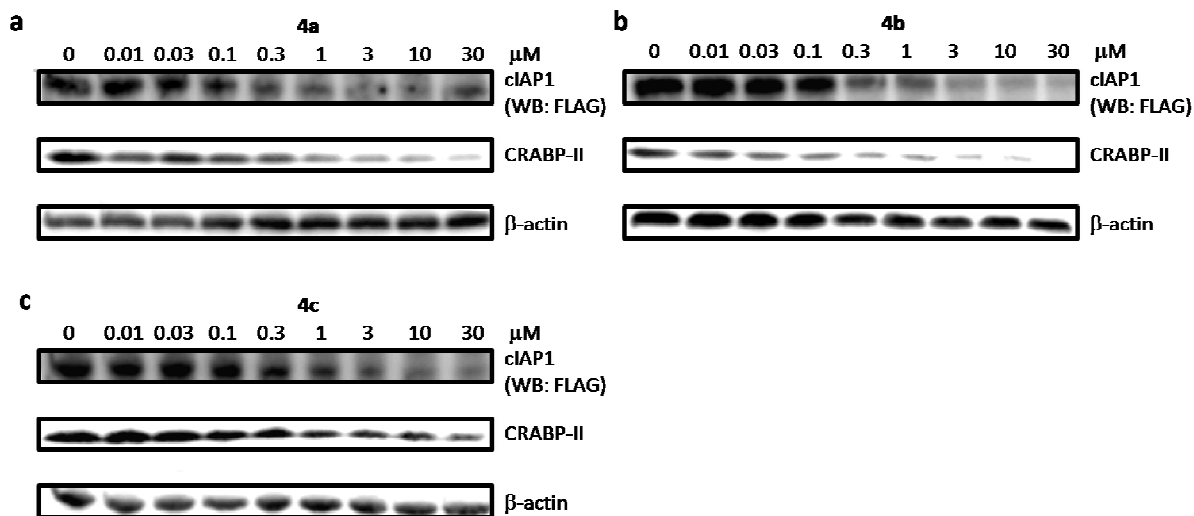


Figure S3. Western blot detection of CRABP-II and cIAP1 levels in FLAG-cIAP1 HT1080 cells after 6-hour treatment with each reagent. (a) Treatment with compound **4a**. (b) Treatment with compound **4b** (c) Treatment with compound **4c**.

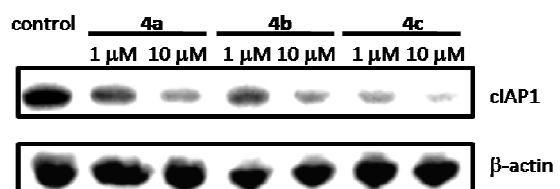


Figure S4. Western blot detection of cIAP1 levels in MOLT-4 cells after 16-hour treatment with **4**.

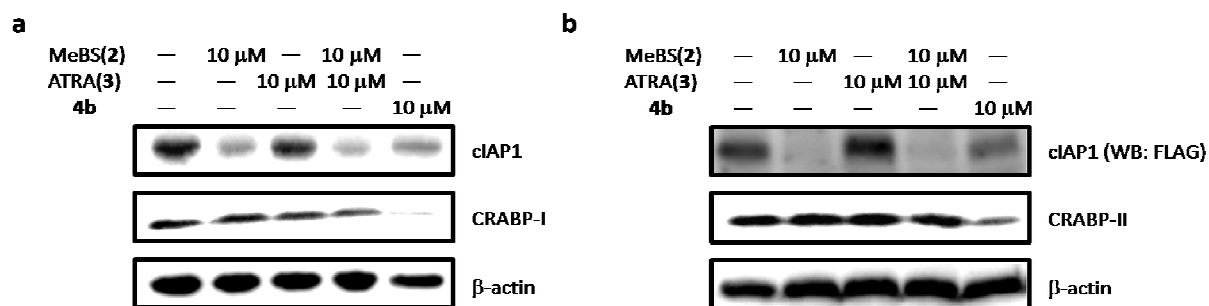


Figure S5. Effect of combinations of MeBS (**2**) and ATRA (**3**). (a) Western blot detection of CRABP-I and cIAP1 levels in MOLT-4 cells after 16-hour treatment with each reagent. MOLT-4 cells were treated with 10 μ M MeBS (**2**), 10 μ M ATRA (**3**) or 10 μ M **4b**. (b) Western blot detection of CRABP-II and cIAP1 levels in FLAG-cIAP1 HT1080 cells after 6-hour treatment with each reagent. HT1080 cells expressing FLAG-tagged cIAP1 were treated with 10 μ M MeBS (**2**), 10 μ M ATRA (**3**) or 10 μ M **4b**.

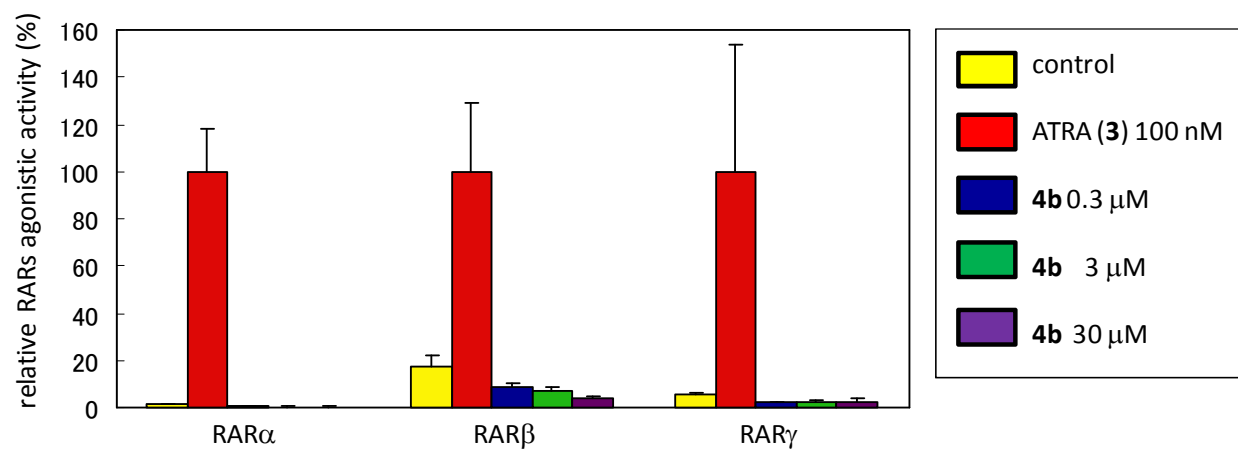


Figure S6. Relative RARs agonistic activity of compound **4b** for ATRA (**3**). Results are presented as means \pm S.E.M. (performed in triplicate).

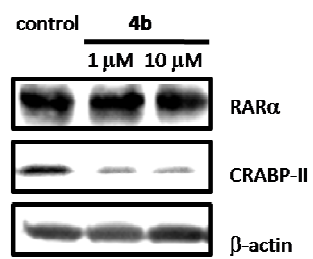


Figure S7. Western blot detection of RAR α levels in HT1080 cells after 6-hour treatment with **4b**.

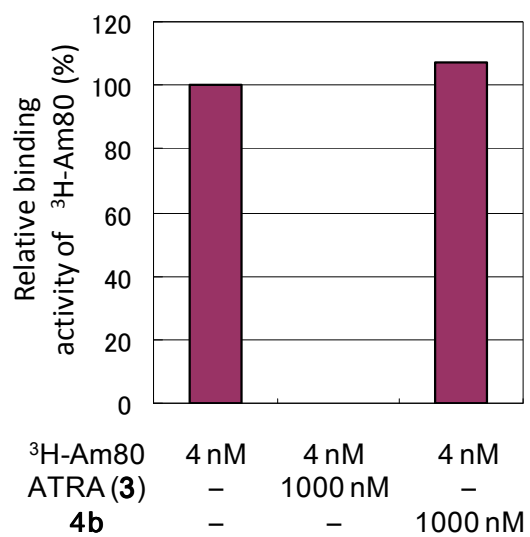


Figure S8. RARs binding activity of compound **4b**..

Complete Reference 35c.

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