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Biological activities of retinoidal γ-hydroxybutenolides in cancer cell apoptosis and differentiation

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Abstract—Retinoidal γ-hydroxybutenolides **2a**–**d** having various lengths of conjugated double bond were prepared in three steps from the corresponding aldehyde **4**. Their biological activities were then measured. Of these compounds, only compound **2c** possessing a structure similar to that of retinoic acid showed differentiation-inducing activity and very strong apoptosis-inducing activity in HL-60 cells.

1. Introduction

A number of retinoids as chemopreventive and chemotherapeutic agents have been reported to exhibit potent activities in the inhibition of uncontrolled cell growth, induction of apoptosis, and promotion of differentiation in cancer cells.[1] Natural retinoids, such as all-*trans* retinoic acid (ATRA) and 9-*cis* retinoic acid (9CRA), exert their biological action through their binding to and activation of specific retinoic acid nuclear receptors (RARs) and retinoid X nuclear receptors (RXRs); these receptors, when formed, complex with the ligand and bind to retinoid response elements (RAREs and RXREs), modulating gene expression.[2] On the other hand, some novel retinoid-related compounds display immense potential as therapeutic agents in tumor treatment.[3] Some of them do not fit into the classical concept of ligand–receptor interaction, but exert a growth-regulatory or apoptogenic activity that is not receptor-mediated.[4]

Previously, one of the authors had found[5] that the novel retinoidal γ-hydroxybutenolide **1** and its analogues containing compound **2a** had an inhibitory effect on the DNA synthesis of mouse neuroblastoma N18TG-2 cells. As shown in Figure 1, γ-hydroxybutenolide **1** is the equivalent of a formyl carboxylic acid **1′**, which has a structure similar to that of retinoic acid having a formyl group at the C-13 position. During this time, we synthesized γ-hydroxybutenolides **2a**–**d** having various lengths of conjugated double bond and investigated their biological activities in human promyelocytic leukemia (HL-60) cells.

2. Chemistry

Known compounds **2a** and **2b** (Fig. 1) were obtained by a method previously reported.[6] Related compounds **2c** and **2d** were synthesized, according to the method[6] with modifications as shown in Scheme 1.

Aldol condensation of the aldehydes **4c**[7] and **4d**[7] with pyruvic aldehyde dimethyl acetal using sodium hydride as a base afforded the acetal-ketones **5c** (93%) and **5d** (84%), respectively. Emmons–Horner reaction of these ketones with triethyl phosphonoacetate in the presence of *n*-BuLi gave isomeric mixtures (2*E*:2*Z* ~2:3) of esters **6c** (81%) and **6d** (79%), respectively, which without separation were treated with aqueous 30% H_2SO_4 and a catalytic amount of iodine in refluxing 1,4-dioxane. In these reactions, hydrolysis, isomerization of the double

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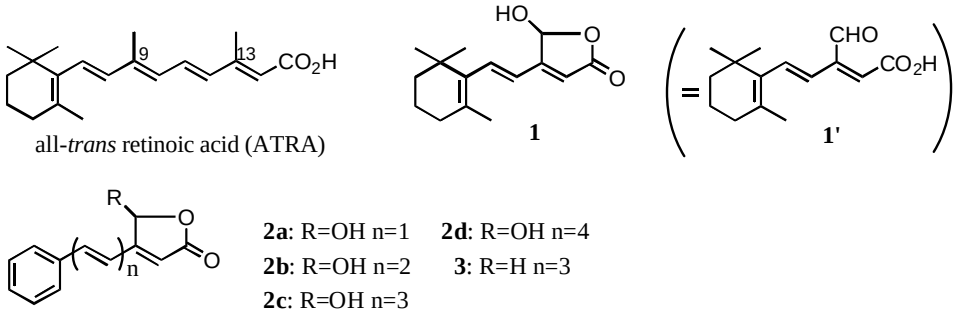
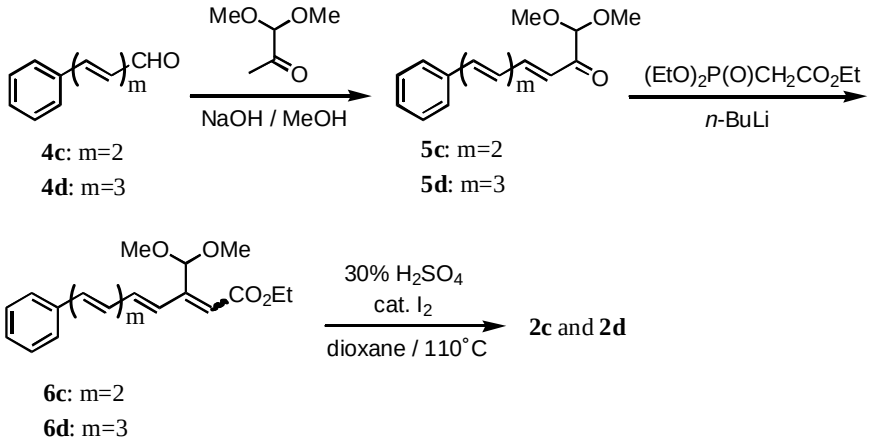


Figure 1. Chemical structures of retinoic acid and butenolides **1**, **2a**–**d**, and **3**.



Scheme 1. Synthesis of compounds **2c** and **2d**.

bond at C-2 position in **6c** and **6d**, and cyclization took place in one pot to give γ-hydroxybutenolides **2c** (42%) and **2d** (16%), respectively.

3. Biological evaluation

Butenolides were initially tested for their apoptosis- and differentiation-inducing activities in HL-60 cells. As shown in Figure 2A, the γ-hydroxybutenolides **2c**, including the same conjugated double bond chain as ATRA, exhibited an approximately 3-fold higher apoptosis-inducing activity than those of ATRA and 9CRA, while other butenolides were virtually inactive. The apoptosis-inducing potency of **2c** at 10^{-6} M was almost compatible to that of staurosporine, a well-known potent inducer of apoptosis in HL-60 cells (data not shown). Like staurosporine, **2c** began to induce apoptosis at 6 h and reached the maximum at 12 h after the treatment, while those activities of ATRA and 9CRA showed up at 48 h and became prominent at 72 h after the treatment (data not shown). It is interesting to note that the induction of apoptosis by **2c** was not dose dependent and **2c** displayed a very narrow apoptosis-inducing window such as that inducible at a concentration of 4×10^{-7} M or higher and non-inducible at a concentration of 2×10^{-7} M or lower, as shown in Figure 2B. These results suggest that the mechanism by which **2c** induces apoptosis of HL-60 cells may be different from a RAR/RXR-mediated differentiation mechanism. Since compound **3**, which does not have any hydroxy group at the γ-position, completely lacked the apoptosis-inducing ability and other butenolides **2a**, **2b**, and **2d**, which have

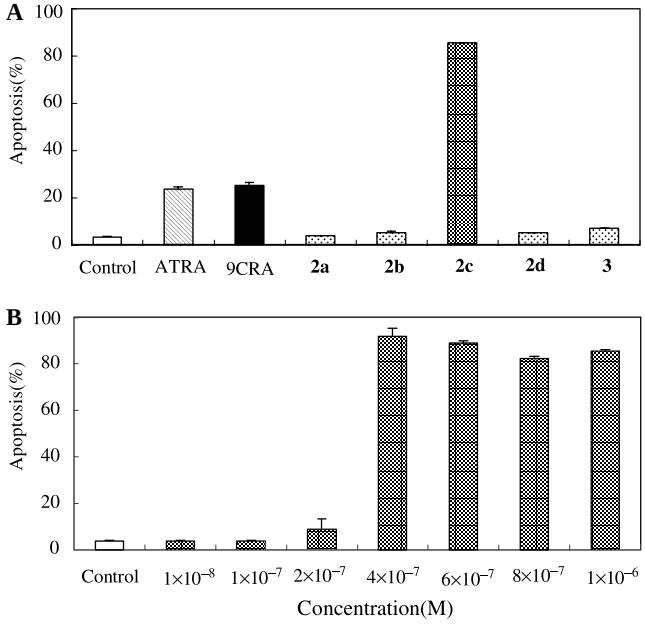


Figure 2. Effect of butenolides on apoptosis. (A) Percentage of apoptosis peak in culture of HL-60 cells treated with vehicle, ATRA, 9CRA, or butenolides **2a**–**d** and **3** at 10^{-6} M. (B) Dose–response columns for γ-hydroxybutenolide **2c** induced apoptosis in cultured HL-60 cells.

different lengths of conjugated double bond, also lacked this ability, the apoptosis-inducing mechanism by **2c** is probably highly structure specific.

To examine the differentiation-inducing activity of butenolides, cell surface CD11b antigen expression as a marker of granulocyte/monocyte/macrophage differentiation and CD14 antigen expression, as a marker of monocyte-associated antigen, were measured. As shown in Figure 3, both ATRA and 9CRA induced CD11b antigen expression in a dose-dependent manner but failed to induce CD14 antigen expression, while **2c** induced both CD11b and CD14 antigen expression at 10^{-6} M. Other butenolides had no effect on both antigens expression. These results are consistent with the results of apoptosis-inducing activity, suggesting again that **2c** modulates HL-60 cell apoptosis and differentiation in a manner independent from RAR/RXR signaling pathway.

To investigate the mechanism by which **2c** modulates apoptosis and differentiation of HL-60 cells and whether RAR or RXR is involved in this process, we generated reporter plasmids including a human RARβ gene RARE or a rat CRABP II gene RXRE and measured the transactivation activity of butenolides in the MG-63 cells. As shown in Figure 4, both ATRA and 9CRA at 10^{-6} M remarkably enhanced RARβ/RARE-mediated gene expression, while all the butenolides, including **2c**, had no such effect. The result suggests that the induction of CD11b and CD14 antigen expression by **2c** may be regulated by a RAR/RARE-independent mechanism. As shown in Figure 5, 9CRA was the most potent modulator of RXRα/RXRE-mediated gene expression among the compounds tested and ATRA was approximately half as potent as 9CRA. Interestingly, butenolides, **2c** and **3**, also induced RXRα/RXRE-mediated gene expression. As mentioned above, compound **3** lacked the ability to induce HL-60 cell apoptosis and differentiation. To examine the binding potency of butenolides to RXRα protein, we conducted an in-cell binding assay using a human RXRα-GAL4 reporter plasmid in MG-63 cells. As shown in Figure 6, ATRA and 9CRA exhibited similar RXRα-binding potencies, as observed in enhancing RXRα/RXRE-mediated gene expression, as shown in Figure 5. The RXRα-binding affinities of butenolides **2c** and **3** was found to be approximately 1.3-fold higher, but not statistically significant, than those of control (ethanol). Based on these RAR/RXR transactivation properties of butenolides, compounds **2c** and **3**, having the same length of conjugated double bond chain as ATRA, appear to conserve the basic property to bind RXR and weak potency to modulate RXR/RXRE-mediated gene expression. In

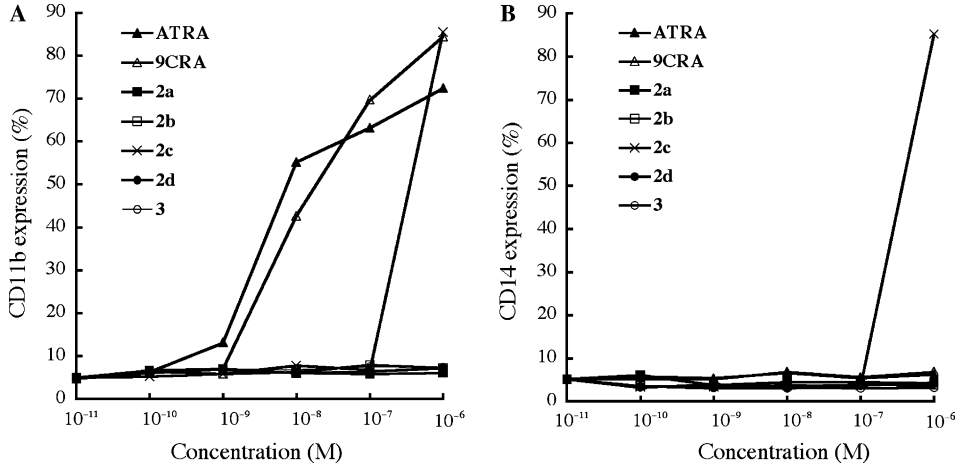


Figure 3. Effect of ATRA, 9CRA, and butenolides **2a–d** and **3** on cell surface CD11b antigen (A) and CD14 antigen (B) in HL-60 cells.

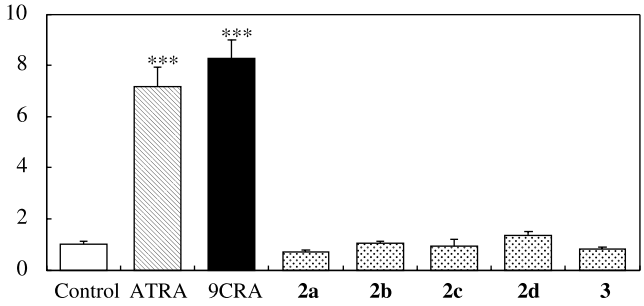


Figure 4. Transcriptional potency of ATRA, 9CRA, and butenolides **2a–d** and **3** (10^{-6} M) on human RARβ gene RARE in MG63 cells. *** $P < 0.001$.

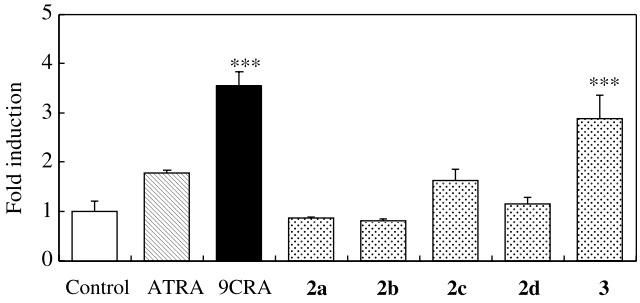


Figure 5. Transcriptional potency of ATRA, 9CRA, and butenolides **2a–d** and **3** (10^{-6} M) on rat CRABP II gene RXRE in MG63 cells. *** $P < 0.001$.

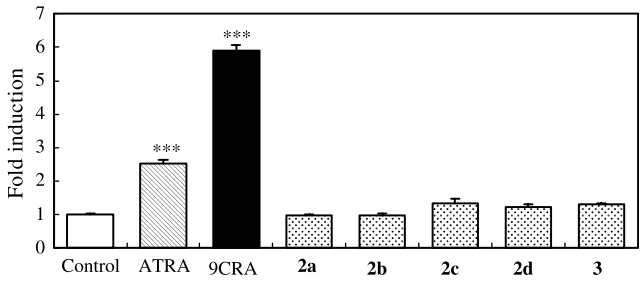


Figure 6. Transcriptional potency of ATRA, 9CRA, and butenolides **2a–d** and **3** (10^{-6} M) on human RXRα-GAL4 expression gene in MG63 cells. $^{***}P < 0.001$.

addition, the present study clearly indicates that, besides apoptogenic activity, **2c** has a unique property of differentiating HL-60 cells into monocytes through a unique mechanism that did not involve retinoid nuclear receptors. At present, we have no explanation on the details of this nuclear receptor-independent mechanism; however, there are a number of studies supporting a retinoid receptor-independent mechanism of apoptosis and differentiation induction in various cancer cell lines (see References).

Hsu et al.[4a] reported that CD437, a novel atypical retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid, inhibits the proliferation of a ATRA-resistant leukemic cell line HL-60R, a virtually RAR-negative cell line, and induces apoptosis within 2 h after the treatment both by cleavage and activation of the CPP32 protease, and cleavage of poly(ADP-ribose) polymerase, followed by the cleavage of bcl-2 and internucleosomal DNA degradation. Since CD437 does not bind to or transactivate RAR and RXR, the mechanism by which CD437 mediates its action seems to be independent from a RAR/RXR-dependent signaling pathway. How CD437 mediates its action remains to be fully defined, although there could be several possible mechanisms raised by speculating that CD437 activates mitochondrial apoptogenic signaling pathway,[8] death receptor pathway,[9] and transcription factors related to proliferation and apoptosis.[10]

Retinoylation, namely retinoic acid acylation, can be another possible candidate to explain about this RAR/RXR-independent mechanism.[11] Takahashi et al.[11] reported that vimentin, an intermediate filament protein, was the most prominent retinoylated protein in HL-60 cells and the extent of this retinoylation depends on the initial concentration of ATRA in a saturable manner. Furthermore, the concentration–effect relationship for ATRA-induced differentiation and for retinoylation was similar. They also found that different retinoylated proteins in various cell types are consistent with the pleiotropic effects of ATRA. Based on this finding, compound **2c** may serve to retinoylate vimentin or other proteins, resulting in inducing apoptosis and differentiation in HL-60 cells.

In summary, we have synthesized retinoidal butenolides, and evaluated their apoptogenic and differentiation-inducing effects on HL-60 cells. Among them, **2c** was found to possess a unique RAR/RXR-independent biological property, although the mechanism by which **2c** mediates its action remains to be defined. Chemotherapy has been highly effective in inducing remission in acute myelogenous leukemia. ATRA has been found to be effective for the treatment of a patient with acute promyelocytic leukemia. However, there are several limitations on the long-term use of ATRA for chemotherapy. They involve rapid metabolic clearance and side effects including skeletal abnormalities, mucocutaneous toxicity, hyperglyceridemia, hypothyroidism, and teratogenesis. Like **2c**, retinoids lacking RAR/RXR-mediated signaling pathway would be a unique and useful candidate for development of cancer chemotherapeutic agents.

4. Experimental

4.1. General methods

Melting points (mp) were measured on a micro melting point apparatus (Yanagimoto) and are uncorrected. UV spectra were recorded on a JASCO Ubest-55 instrument. IR spectra were measured on a Perkin–Elmer FT-IR spectrometer, model Paragon 1000. ^{1}H NMR spectra were determined on a Varian Gemini-300 or a Varian VXR-500 superconducting FT-NMR spectrometer and the chemical shifts were referenced to tetramethylsilane. Mass spectra were taken on a Hitachi M-4100 spectrometer. Column chromatography (CC) was performed on silica gel (Merck Art. 7734).

4.1.1. Preparation of ketones 5c and 5d. To a solution of the aldehyde **4c**[7] (1.02 g, 6.46 mmol) and pyruvic aldehyde dimethyl acetal (1.52 g, 12.9 mmol) in MeOH (2 mL) was added NaOH (1.0 M in MeOH; 0.5 mL) at rt and the mixture was stirred for a further 30 min. After being quenched with saturated aq NH_4Cl, the mixture was extracted with AcOEt. The extracts were washed with brine, dried, and evaporated. The resulting crude product was purified by CC (AcOEt/hexane, 1:3) to provide the ketone **5c** (1.55 g, yield: 93%) as pale yellow crystals. Compound **5d** was similarly prepared (yield: 84%) from the aldehyde **4d**.[7]

(3*E*,5*E*,7*E*)-1,1-Dimethoxy-8-phenylocta-3,5,7-trien-2-one (**5c**): Mp 46–47 °C (from ether/hexane); IR $\nu_{max}(CHCl_3)$ cm^{-1}: 1687, 1600, 1580, 1567; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.43 (6H, s), 4.69 (1H, s), 6.47 (1H, dd, J = 14, 11.5 Hz), 6.52 (1H, d, J = 15 Hz), 6.76 (1H, d, J = 15 Hz), 6.81 (1H, dd, J = 15, 10 Hz), 6.90 (1H, dd, J = 15, 10 Hz), 7.27 (1H, t, J = 7 Hz), 7.34 (2H, t, J = 7 Hz), 7.44 (2H, d, J = 7 Hz), 7.50 (1H, dd, J = 15, 11.5 Hz). UV λ_{max}(EtOH) nm: 364; EI-MS m/z: 258.1262 (Calcd for $C_{16}H_{18}O_3$: 258.1255).

(3*E*,5*E*,7*E*,9*E*)-1,1-Dimethoxy-10-phenyldeca-3,5,7,9-tetraen-2-one (**5d**): Mp 80–81 °C (from EtOH); ^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.42 (6H, s), 4.68 (1H, s), 6.41 (1H, dd, J = 15, 11.5 Hz), 6.44 (1H, dd, J = 15, 11 Hz), 6.49 (1H, d, J = 15.5 Hz), 6.61 (1H, dd, J = 15, 10.5 Hz), 6.68 (1H, d, J = 15.5 Hz), 6.75 (1H, dd, J = 15, 11 Hz), 6.87 (1H, dd, J = 15.5, 10.5 Hz), 7.25

(1H, t, $J = 7$ Hz), 7.33 (2H, t, $J = 7$ Hz), 7.42 (2H, d, $J = 7$ Hz), 7.47 (1H, dd, $J = 15.5$, 11.5 Hz); UV λ_{max} (EtOH) nm: 390; EI-MS *m/z*: 284.1426 (Calcd for $C_{18}H_{20}O_3$: 284.1412); Anal. Calcd for $C_{18}H_{20}O_3$: C, 76.03; H, 7.09. Found: C, 75.78; H, 7.22.

4.1.2. Preparation of esters 6c and 6d. A solution of *n*-BuLi (1.58 M in hexane; 5.78 mL) was added to a stirred solution of ethyl (diethoxyphosphoryl)acetate (2.00 g, 8.93 mmol) in dry THF (25 mL) at 0 °C and the mixture was stirred for a further 15 min. To this mixture was added dropwise a solution of the ketone **5c** (1.52 g, 5.89 mmol) in dry THF (10 mL) at 0 °C and the mixture was heated for 1 h at 50 °C. After being quenched with saturated aq NH_4Cl, the mixture was extracted with AcOEt. The extracts were washed with brine, dried, and evaporated. The resulting crude product was purified by CC (THF/hexane, 1:4) to provide an isomeric mixture (*E*/*Z* ~2:3) of the ester **6c** (1.57 g, yield: 81%). Purification of a part of the isomeric mixture by preparative HPLC [LiChrosorb Si 60 (7 μm) 1.0 × 30 cm; AcOEt/hexane, 12:88] provided each pure isomer as yellow solids. Compound **6d** was similarly prepared (yield: 79%) from the ketone **5d**.

Ethyl (2*E*,4*E*,6*E*,8*E*)-3-(dimethoxymethyl)-9-phenylnona-2,4,6,8-tetraenoate (*E*-**6c**): IR $\nu_{max}(CHCl_3)$ cm^{-1}: 1703, 1622, 1598, 1583. ^1H NMR (300 MHz, $CDCl_3$) δ: 1.31 (3H, t, $J = 7$ Hz), 3.32 (6H, s), 4.21 (2H, q, $J = 7$ Hz), 5.14, 6.08 (each 1H, s), 6.48 (1H, dd, $J = 14.5$, 10.5 Hz), 6.59 (1H, dd, $J = 14.5$, 10.5 Hz), 6.62 (1H, d, $J = 15.5$ Hz), 6.88 (1H, dd, $J = 15.5$, 10.5 Hz), 6.97 (1H, dd, $J = 15.5$, 10.5 Hz), 7.23 (1H, t, $J = 7.5$ Hz), 7.32 (2H, t, $J = 7.5$ Hz), 7.42 (2H, d, $J = 7.5$ Hz), 7.62 (1H, d, $J = 15.5$ Hz). UV λ_{max}(EtOH) nm: 367. EI-MS *m/z*: 328.1682 (Calcd for $C_{20}H_{24}O_4$: 328.1674).

Ethyl (2*Z*,4*E*,6*E*,8*E*)-3-(dimethoxymethyl)-9-phenylnona-2,4,6,8-tetraenoate (*Z*-**6c**): IR $\nu_{max}(CHCl_3)$ cm^{-1}: 1702, 1612, 1598, 1579. ^1H NMR (300 MHz, $CDCl_3$) δ: 1.31 (3H, t, $J = 7$ Hz), 3.47 (6H, s), 4.19 (2H, q, $J = 7$ Hz), 6.04, 6.18 (each 1H, s), 6.25 (1H, d, $J = 15.5$ Hz), 6.40 (1H, dd, $J = 14.5$, 11 Hz), 6.57 (1H, dd, $J = 14.5$, 11 Hz), 6.62 (1H, d, $J = 15.5$ Hz), 6.85 (1H, dd, $J = 15.5$, 11 Hz), 7.11 (1H, dd, $J = 15.5$, 11 Hz), 7.23 (1H, t, $J = 7$ Hz), 7.32 (2H, t, $J = 7$ Hz), 7.41 (2H, d, $J = 7$ Hz). UV λ_{max}(EtOH) nm: 367. EI-MS *m/z*: 328.1680 (Calcd for $C_{20}H_{24}O_4$: 328.1674).

Ethyl (2*E*,4*E*,6*E*,8*E*,10*E*)-3-(dimethoxymethyl)-11-phenylundeca-2,4,6,8,10-pentaenoate (*E*-**6d**): IR $\nu_{max}(CHCl_3)$ cm^{-1}: 1702, 1613, 1600, 1567. ^1H NMR (500 MHz, $CDCl_3$) δ: 1.30 (3H, t, $J = 7$ Hz), 3.32 (6H, s), 4.20 (2H, q, $J = 7$ Hz), 5.13, 6.06 (each 1H, s), 6.42 (1H, dd, $J = 15$, 10.5 Hz), 6.43 (1H, dd, $J = 14.5$, 10 Hz), 6.48 (1H, dd, $J = 14.5$, 10 Hz), 6.51 (1H, dd, $J = 15$, 10 Hz), 6.61 (1H, d, $J = 15.5$ Hz), 6.87 (1H, dd, $J = 15.5$, 10 Hz), 6.95 (1H, dd, $J = 16$, 10.5 Hz), 7.22 (1H, t, $J = 7.5$ Hz), 7.32 (2H, t, $J = 7.5$ Hz), 7.41 (2H, d, $J = 7.5$ Hz), 7.60 (1H, d, $J = 16$ Hz). UV λ_{max}(EtOH) nm: 389. EI-MS *m/z*: 354.1835 (Calcd for $C_{22}H_{26}O_4$: 354.1829).

Ethyl (2*Z*,4*E*,6*E*,8*E*,10*E*)-3-(dimethoxymethyl)-11-phenylundeca-2,4,6,8,10-pentaenoate (*Z*-**6d**): IR $\nu_{max}(CHCl_3)$ cm^{-1}: 1702, 1594, 1565. ^1H NMR (500 MHz, $CDCl_3$) δ: 1.31 (3H, t, $J = 7$ Hz), 3.46 (6H, s), 4.19 (2H, q, $J = 7$ Hz), 6.02, 6.18 (each 1H, s), 6.23 (1H, d, $J = 15.5$ Hz), 6.33 (1H, dd, $J = 15$, 11 Hz), 6.41 (1H, dd, $J = 14.5$, 10.5 Hz), 6.48 (1H, dd, $J = 14.5$, 10 Hz), 6.49 (1H, dd, $J = 15$, 10.5 Hz), 6.60 (1H, d, $J = 15.5$ Hz), 6.85 (1H, dd, $J = 15.5$, 10 Hz), 7.09 (1H, dd, $J = 15.5$, 11 Hz), 7.22 (1H, t, $J = 7.5$ Hz), 7.31 (2H, t, $J = 7.5$ Hz), 7.40 (2H, d, $J = 7.5$ Hz). UV λ_{max}(EtOH) nm: 388. EI-MS *m/z*: 354.1831 (Calcd for $C_{22}H_{26}O_4$: 354.1829).

4.1.3. Preparation of γ-hydroxybutenolides 2c and 2d. To a solution of the ester **6c** (661 mg, 2.02 mmol) in dioxane (6 mL) were added aq 30% H_2SO_4 (2 mL) and I_2 (2 mg), and the mixture was refluxed for 1 h. After cooling, the reaction mixture was diluted with ether, washed with brine, dried, and evaporated. The residue was purified by CC (acetone/hexane, 3:7) to give the butenolide **2c** (217 mg, yield: 42%) as yellow solids. Compound **2d** was similarly prepared (yield: 16%) from the ester **6d**.

5-Hydroxy-4-[(1*E*,3*E*,5*E*)-6-phenylhexa-1,3,5-trienyl]-2(5*H*)-furanone (**2c**): Mp 185–187 °C (from acetone/hexane). IR ν_{max}(nujol) cm^{-1}: 3269, 3165, 1732, 1711. ^1H NMR (500 MHz, acetone-d_6) δ: 2.86 (1H, s), 6.02 (1H, s), 6.36 (1H, s), 6.59 (1H, d, $J = 15.5$ Hz), 6.61 (1H, dd, $J = 15$, 11 Hz), 6.80 (1H, dd, $J = 15$, 11 Hz), 6.82 (1H, d, $J = 15.5$ Hz), 7.08 (1H, dd, $J = 15.5$, 11 Hz), 7.13 (1H, dd, $J = 15.5$, 11 Hz), 7.28 (1H, t, $J = 7.5$ Hz), 7.36 (2H, t, $J = 7.5$ Hz), 7.53 (2H, d, $J = 7.5$ Hz). UV λ_{max}(EtOH): 368 nm. UV λ_{max}(THF): 368 nm (ε 61,000). EI-MS *m/z*: 254.0942 (Calcd for $C_{16}H_{14}O_3$: 254.0942).

5-Hydroxy-4-[(1*E*,3*E*,5*E*,7*E*)-8-phenylocta-1,3,5,7-tetraenyl]-2(5*H*)-furanone (**2d**): Mp 201–203 °C (from acetone/hexane). IR ν_{max}(nujol) cm^{-1}: 3269, 3165, 1737, 1710. ^1H NMR (300 MHz, DMSO-d_6) δ: 6.12 (1H, s), 6.29 (1H, br s), 6.53 (1H, d, $J = 15.5$ Hz), 6.49–6.60 (2H, m), 6.67 (1H, dd, $J = 15$, 10.5 Hz), 6.72 (1H, dd, $J = 15$, 11 Hz), 6.74 (1H, d, $J = 15.5$ Hz), 6.99 (1H, dd, $J = 15.5$, 11 Hz), 7.06 (1H, dd, $J = 15.5$, 10.5 Hz), 7.26 (1H, t, $J = 7.5$ Hz), 7.35 (2H, t, $J = 7.5$ Hz), 7.51 (2H, d, $J = 7.5$ Hz), 7.86 (1H, br s). UV λ_{max}(EtOH): 392 nm (ε 66,500). UV λ_{max}(THF): 391 nm (ε 71,000). EI-MS *m/z*: 280.1108 (Calcd for $C_{18}H_{16}O_3$: 280.1099).

4.1.4. Preparation of butenolide 3. To a solution of γ-hydroxybutenolide **2c** (212 mg, 0.83 mmol) in THF (3 mL) and MeOH (3 mL) was added $NaBH_4$ (30 mg, 0.80 mmol) at 0 °C, and the reaction mixture was stirred at rt for 20 min. After being quenched with saturated aq NH_4Cl, the mixture was extracted with ether. The extracts were washed with brine, dried, and evaporated. The resulting crude product was purified by CC (acetone/hexane, 1:2) to give the compound **3** (113 mg, yield: 57%) as yellow solids.

4-[(1*E*,3*E*,5*E*)-6-phenylhexa-1,3,5-trienyl]-2(5*H*)-furanone (**3**): IR $\nu_{max}(CHCl_3)$ cm^{-1}: 1780, 1746, 1628, 1598. ^1H

NMR (300 MHz, $CDCl_3$) δ: 5.01 (2H, s), 5.92 (1H, s), 6.43 (1H, dd, J = 14.5, 10.5 Hz), 6.50 (1H, d, J = 15.5 Hz), 6.62 (1H, dd, J = 15.5, 10.5 Hz), 6.65 (1H, dd, J = 15.5, 10.5 Hz), 6.71 (1H, d, J = 15.5 Hz), 6.89 (1H, dd, J = 15.5, 10.5 Hz), 7.27 (1H, t, J = 7.5 Hz), 7.34 (2H, t, J = 7.5 Hz), 7.44 (2H, d, J = 7.5 Hz). UV λ_{max}(MeOH): 365 nm (ε 52,000). EI-MS *m/z*: 238.0999 (Calcd for $C_{16}H_{14}O_2$: 238.0993).

4.2. Biological assay

4.2.1. HL-60 cells and synchronization of cell cycle at S phase by excess amounts of thymidine. HL-60 cells were maintained in continuous culture in RPMI-1640 medium (Nissui Seiyaku Co., Ltd., Tokyo, Japan) supplemented with 10% dextran-coated charcoal-treated fetal calf serum (FCS) (Gibco BRL, Grand Island, NY, USA) and kanamycin (0.06 mg/mL) (Sigma, St. Louis, MO, USA) at 37° C in a humidified atmosphere of 5% CO_2 in air. The doubling time of HL-60 cells was approximately 24 h. For synchronization at S phase, cells (4×10^5 cells/mL) were cultured in 30 mL RPMI-1640 medium supplemented with 2.5 mM thymidine. After washing with Ca, Mg-free phosphate-buffered saline (PBS) [PBS(−)] twice, synchronization of the cell cycle was repeated in the same manner, and the cells thus obtained were used in biological assays.

4.2.2. Flow cytometry. Cells (10^5 cells/well) were placed in 24-well tissue culture plates and cultured for 3 days with retinoids (10^{-10}–10^{-6} M) in RPMI-1640 medium at 37° C in a humidified atmosphere of 5% CO_2 in air. To reduce the effects of contact inhibition, control cells were adjusted to 60–70% confluency at the time of FACS analysis. Each group of cells was collected in PBS(−). Then, the cells were resuspended in PBS(−) containing 0.2% Triton-X and 1 μg/μL RNase, and incubated at 37° C for 1 h. Cells were washed with PBS(−) and incubated with 0.5 mL DNA-staining solution containing propidium iodide (50 μg/mL) at 4° C for 20 min. The cells were analyzed with a flow cytometer equipped with an argon laser (488 nm, Becton Dickinson FACScan™) and cell cycle distribution was analyzed by ModiFiT LT (Verity).

4.2.3. Cell surface antigen expression analysis. Cells (10^5 cells/well) were placed in 24-well tissue culture plates and cultured for 3 days in RPMI-1640 medium with retinoids (10^{-10}–10^{-6} M) under the same conditions as described for flow cytometry. Each group of cells was then collected and washed with PBS(−) once. Then, the cells (2×10^5 cells) were resuspended in 100 μL diluent solution containing 1% bovine serum albumin (BSA) and 1% sodium azide, and incubated with 10 μL human monoclonal FITC-conjugated CD11b antibody and CD14 antibody (Sigma) for 30 min at room temperature. The cells were washed once with diluent solution and then fixed in 300 μL PBS(−) containing 2% paraformaldehyde. Fluorescence was detected on a Becton Dickinson FACScan™ at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. Results were recorded as the mean fluorescence index, which is a product of % fluorescence and mean fluorescence intensity, with 10^4 cells being counted per treatment.

4.2.4. Transfection and luciferase activity assay. Human osteosarcoma MG-63 cells, which are positive for RXR gene expression, were maintained in Dulbecco's modification Eagle's medium (Gibco BRL) supplemented with 1% penicillin, 1% streptomycin, and 10% dextran-coated charcoal-treated FCS (Gibco BRL). The day before transfection, cells were seeded on six-well culture plates at a density of 2×10^5 cells/well so that they reached confluence the day of transfection. Cells were transfected with 1.0 μg of a one-hybrid plasmid (pM vector, Promega Corp., Madison, WI, USA) containing a human RXR cDNA connected with a yeast GAL4 DNA-binding domain cDNA (GAL-DBD), 0.5 μg of luciferase reporter plasmid (pGVP2 vector, Toyo Ink Co., Ltd.) containing a GAL-4 binding site (GAL-BS) and a pRL-CMV vector as an internal control using the Tfx-50 reagent (Promega Corp.).

Human osteosarcoma MG-63 cells, which are positive for RAR gene expression, were maintained in Dulbecco's modification Eagle's medium (Gibco BRL) supplemented with 1% penicillin, 1% streptomycin, and 10% dextran-coated charcoal-treated FCS (Gibco BRL).The day before transfection, cells were seeded on six-well culture plates at a density of 2×10^5 cells/well so that they became confluent the day of transfection. The retinoid responsive luciferase reporter constructs human RARβ-$RARE_3$-SV40-Luc and rat CRBP II-RXRE-SV40-Luc were generated by cloning three copies of the retinoic acid response element (RARE) from the RARβ promoter (−59/−33: GGGTAAAGTT CACCGAAAGTTCACTCG) or the RXRE from the rat CRBP II promoter (−639/−605: GCTGTCA CAGGTCACAGGTCACAGGTCACAGTTCA) in pGL3 vector.[12] The pRL-CMV vector served as an internal control using the Tfx-50 reagent. After transfection, cells were incubated with retinoids (10^{-6} M) for 2 days. luciferase activities of the cell lysates were measured with a luciferase assay system (Toyo Ink Co., Ltd.), according to the manufacturer's instructions. Transactivation measured by luciferase activity was standardized with the luciferase activity of the same cells determined by the Sea Pansy luciferase assay system as a control (Toyo Ink Co. Ltd.). Each set of experiments was repeated at least three times, and the results are presented in terms of fold induction as means ± SE.

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