Synthesis and Characterization of Heteroarotinoids Demonstrate Structure Specificity Relationships

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Heteroarotinoids are synthetic retinoids derived from *trans*-retinoic acid and the arotinoid structures and include a heteroatom in a five- or six-membered cyclic ring. This is the first systematic study of influences of the heteroatom, ring size, number of aryl groups, and terminal side chain on retinoid receptor specificity. Two new heteroarotinoids were synthesized and characterized. Although all heteroarotinoids activated RAR receptors, two dominant associations between structure and specificity were identified across all compounds. The six-membered ring conferred increased RARβ specificity over the five-membered ring. The sulfur atom conferred greater specificity for RARγ than the oxygen atom. RARα specificity was attenuated by a combination of influences from the heteroatom and aryl groups. In summary, the heteroatom and cyclic ring size exerted dominant effects, while the number of aryl rings and terminal side chain had attenuating effects on retinoid receptor specificity of heteroarotinoids.

Introduction

Heteroarotinoids are a class of retinoids that have demonstrated significant promise as anticancer agents. The potential of these compounds to prevent or inhibit transformation is suggested by their inhibition of ornithine decarboxylase (ODC) induction.[1,2] ODC is transiently upregulated upon growth stimulation and catalyzes the synthesis of polyamines required for DNA synthesis. The role of ODC in cellular transformation is demonstrated by the constitutive ODC activity observed in transformed cells and by the transformation of NIH3T3 cells induced by overexpression of ODC alone.[3] Heteroarotinoids have also been shown to induce differentiation in human leukemia cells and tracheal organ culture (TOC).[1,4−6] In a mouse chemoprevention model, two heteroarotinoids were shown to reduce the number of papilloma tumors initiated by a carcinogen by greater than 90% when administered at doses that induced no observable toxic side effects.[1]

The clinical promise of heteroarotinoids is further suggested by their decreased toxicity in comparison to retinoic acid and arotinoids.[1,4] The reason for the reduced toxicity appears to be due to a single structural alteration, namely inclusion of a heteroatom in the cyclic ring.[4] Heteroarotinoids (Figure 1) are modifications of *trans*-retinoic acid (**1)** and the arotinoid (**2)** structures and include a heteroatom in a six-membered cyclic ring (**3** and **4**, respectively) or in a five-membered cyclic ring (**5** and **6)**. Inclusion of a heteroatom in the cyclic ring of the arotinoid backbone reduces the toxicity 1000-fold, whereas inclusion of a heteroatom in the cyclic ring of *trans*-retinoic acid reduces the toxicity 3-fold.[4]

The molecular mechanism action of heteroarotinoids is most likely through the nuclear retinoid receptors as indicated by transactivation of a retinoic acid response element in treated cells.[4,7,8] In this report, activation of individual retinoid receptors is demonstrated, and the influence of the heteroatom and other structural modifications on the receptor activation and specificity is systematically evaluated.

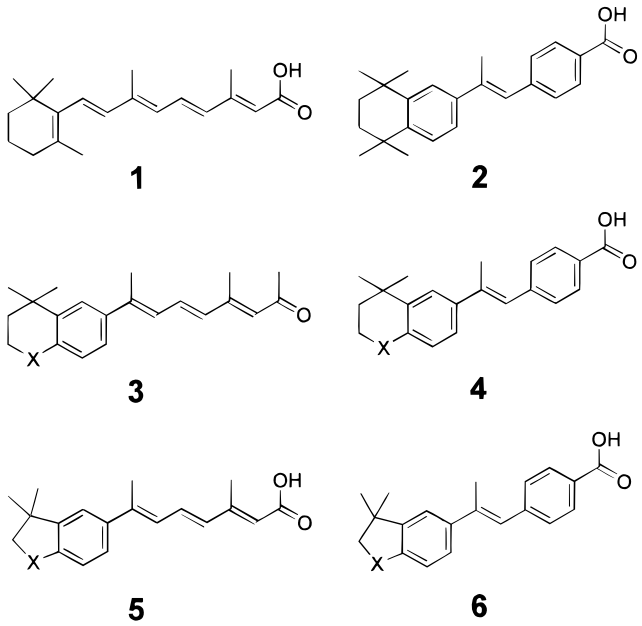

Figure 1. Structure of retinoids and heteroarotinoids.

Results

Chemistry. The syntheses of acid **10** and ester **12** were accomplished via the following reaction sequence (Scheme 1). The Grignard reagent of 5-bromo-2,3-dihydro-3,3-dimethylbenzofuran in dry THF was added to a cool (−40 °C) solution of freshly distilled acetyl

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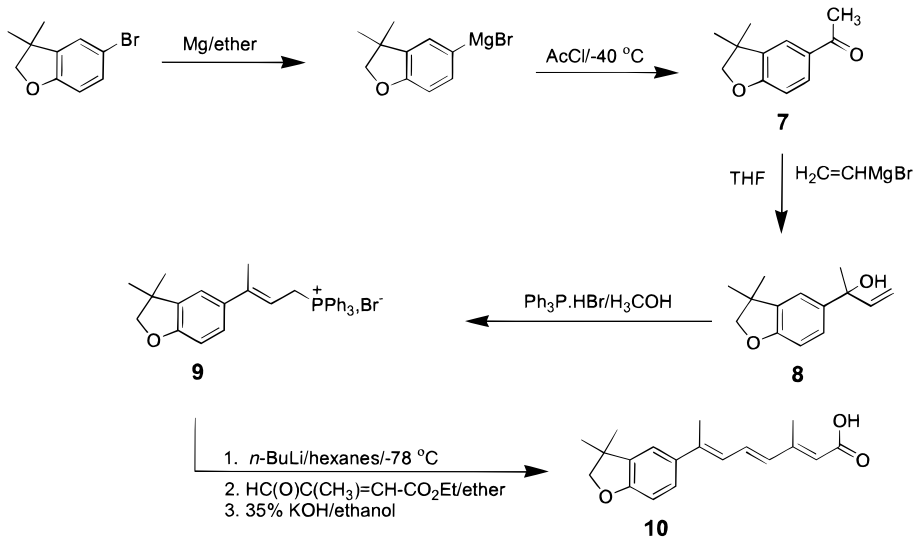
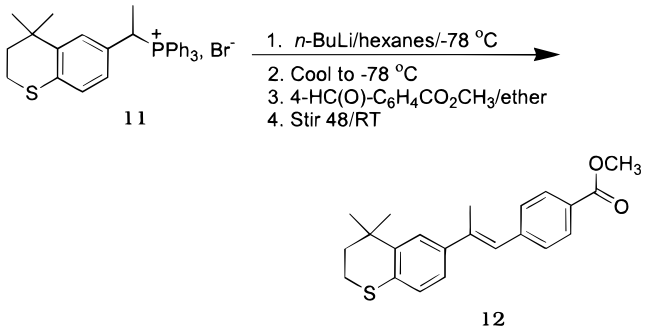
Scheme 1



Scheme 2



chloride under N_2. A standard workup provided ketone **7** (51.6%). Treatment of **7** with vinylmagnesium bromide in the THF under N_2 gave the tertiary alcohol **8** (99%) as an oil. A solution of **8** in methanol was then added to $Ph_3P{\cdot}HBr$ in methanol and led to salt **9** (61%). Generation of the corresponding Wittig reagent from **9** using *n*-butyllithium in hexanes at −78 °C was followed by the addition of ethyl 4-formylcrotonate in dry ether. A standard workup gave an oil which was subjected to treatment with 35% KOH in ethanol. Recrystallization from ethanol of the solid formed gave acid **10** (23%).

Formation of the Wittig reagent of the known salt **11** was followed by the addition of methyl 4-formylbenzoate in ether. The usual workup led to ester **12** (37%) (Scheme 2).

The intermediates obtained in the synthesis of **10**, as well as **10** and **12**, were completely characterized by elemental and spectral analysis as shown in the Experimental Section. All such data were confirming of the structures illustrated.

Biology. The data in Table 1 demonstrates that heteroarotinoids are capable of specifically activating nuclear retinoid receptors. The heteroarotinoids are illustrated and organized in Table 1 such that those compounds differing by only one structural alteration are located adjacent to each other. For each row, the structure in the second column differs from that in the first column only by the size of the cyclic ring, with the exceptions of **12** and **18**, which have different terminal side chains. The receptor specificity is indicated by the potency for each receptor (nM EC_{50} value), and the efficacy of retinoid receptor activation relative to 9-*cis*-retinoic acid is presented in parentheses.

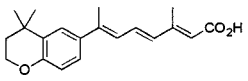
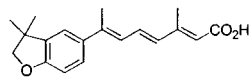
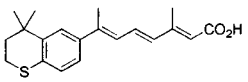
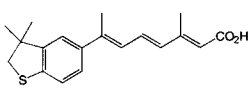
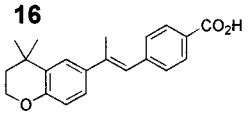
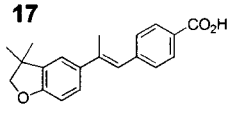
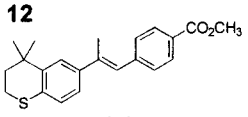
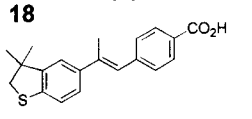
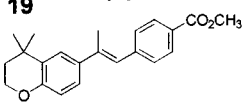
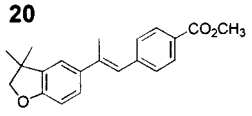
In general, compounds with six-membered rings exhibited greater efficacy than related five-membered ring compounds. Only 3 out of 20 comparisons showed a slightly lower efficacy of the six-membered ring (RARα and RXRα for **13** versus **10**; and RXRα for **14** versus **15**). The six-membered ring compounds also exhibited greater specificity for RARβ as indicated by consistently lower RARβ EC_{50} values in comparison to related five-membered ring compounds (**13** versus **10**, **14** versus **15**, **16** versus **17**, **19** versus **20**). The opposite relationship in **18** versus **12** might be due to an increase in the RARβ EC_{50} value induced by the ester side chain in **12**. Although this is observed for **20** versus **17**, the lower RARβ EC_{50} value of the ester **19** in comparison to the corresponding acid **16** argues against this. A larger number of compounds will need to be evaluated in order to investigate this relationship. No consistent dominant influence of the type of side chain was observed in **19** versus **20** and in **20** versus **17** in this study. The influence of the six- versus five-membered cyclic ring systems on the specificity for the other receptors appeared to be attenuated by other factors.

The sulfur heteroatom was associated with a lower RARγ EC_{50} value in every pair of structurally related compounds (**14** versus **13**, **15** versus **10**, **12** versus **19**, **18** versus **17**). This consistency across all 8 compounds indicates that the type of heteroatom dominates over the influences of other structural modifications. The influence of the sulfur atom on the specificity for the other receptors was attenuated by the monoaryl versus diaryl structure (Table 1).

The number of aryl rings in the structure influenced the efficacy of RXR activation. In contrast to the parent retinoic acid (**1**), monoaryl heteroarotinoid compounds **10**, **13**, **14**, and **15** exhibited significant efficacy but poor specificity for the RXRα receptor. Similar to the parent arotinoid compound **2**, the diaryl heteroarotinoids **12**, **16**, **17**, **18**, **19**, and **20** are poor RXR transactivators.[9]

A combination of the type of heteroatom and the number of aryl groups modified RARα specificity in a consistent manner. In the presence of a sulfur atom,

Table 1. Structure, Potency (nM EC_{50}), and Efficacy (Maximal Response Relative to 9-*cis*-Retinoic Acid) of Heteroarotinoids[a]

Hetero -atom	6-membered cyclic rings	Potency (Efficacy)	5-membered cyclic rings	Potency (Efficacy)	Aryl group
O	13 	RARα 740 (92) RARβ 72 (107) RARγ 39 (111) RXRα 2800 (93)	10 	RARα 800 (96) RARβ 770 (77) RARγ 230 (93) RXRα 2800 (102)	Mono
S	14 	RARα 1400 (103) RARβ 43 (99) RARγ 12 (106) RXRα 2700 (121)	15 	RARα 1000 (89) RARβ 1200 (77) RARγ 8 (66) RXRα 2600 (132)	Mono
O	16 	RARα 1100 (76) RARβ 190 (88) RARγ 200 (76) RXRα NA (5)	17 	RARα 2300 (32) RARβ 320 (50) RARγ 460 (40) RXRα NA (4)	Di
S	12 	RARα 620 (62) RARβ 110 (45) RARγ 84 (65) RXRα NA (2)	18 	RARα 420 (22) RARβ 18 (34) RARγ 30 (44) RXRα NA (1)	Di
O	19 	RARα 1100 (73) RARβ 140 (72) RARγ 330 (71) RXRα NA (6)	20 	RARα NA (20) RARβ 430 (55) RARγ 400 (41) RXRα NA (2)	Di

[a] Compounds differing by only the type of heteroatom are indicated by inverted equal signs. The darker line separates the structures with acid terminal side chains from those with ester terminal side chains.

Table 2. Attenuating Effects of Aryl Groups and Heteroatoms on RARα Specificity

	EC_{50} (nM)	
type of compounds	sulfur heteroatom	oxygen heteroatom
monoaryl	>1000 nM	<1000 nM
diaryl	<1000 nM	>1000 nM

the EC_{50} values for RARα were consistently lower in diaryl compounds than in monoaryl compounds. In the presence of an oxygen atom however, the opposite relationship was observed (Table 2).

Discussion

This paper systematically demonstrates that certain structural modifications of heteroarotinoids can alter the receptor specificity profile. The ability to modulate RARα and RARβ specificity is desirable for the development of anticancer agents because these two receptors are altered in specific types of tumors and are therefore implicated in tumorigenic progression. A direct involvement of RARα in acute promyelocytic leukemia (APL) has been demonstrated by the discovery that the specific t(15;17) translocation associated with APL causes the fusion of the PML gene on chromosome 15 to the RARα gene on chromosome 17.[10−12] RARα specificity has been shown to correlate with the growth inhibitory activity of retinoids in mammary carcinoma cells.[13] RARβ is implicated as a tumor suppressor gene because of its loss or decreased expression in tumors of the lung, oral cavity, and mammary gland[14−16] and has been implicated in both growth inhibition and differentiation.[17−19] In this study, RARα specificity of heteroarotinoids was modulated by both the type of heteroatom and the size of the cyclic ring, while RARβ specificity was greater in six-membered ring compounds than in five-membered ring compounds.

RARγ specificity is an important characteristic to control in topical applications of retinoids since RARγ has been shown to mediate both topical retinoid efficacy and irritation.[20,21] In addition, selectivity for this receptor subtype has been implicated in tumor cell growth inhibition.[22] In this study, the sulfur atom was shown to confer greater RARγ specificity in comparison to the oxygen heteroatom.

The combination of specificity for individual RAR's with specificity for individual RXR's has the potential to increase the potency of retinoids and to decrease the teratogenicity and toxicity of these compounds. While RAR-selective retinoids are much more potent tumor cell growth inhibitors than RXR-selective compounds, the combination of the two results in synergistic effects on growth inhibitory activity.[23] RXR activation is a characteristic that may reduce the teratogenic potential of these compounds, since RXR-specific agonists have been shown to possess much less potent teratogenic activity than RAR-specific agonists.[24,25] In addition, manipulation of receptor specificity may reduce the toxicity of these compounds, since the RAR receptors have been shown to be involved in retinoid-induced hypertriglyceridemia in rats.[26] In this study, the monaryl heteroarotinoid structure exhibited RXR activation

capability, a characteristic not possessed by the parent *trans*-retinoic acid (**1**).

In conclusion, we have demonstrated that a class of retinoid compounds can be structurally altered to increase the specificity for individual retinoid receptors. While the heteroatom and cyclic ring size exerted dominant effects, the number of aryl rings and terminal side chain had attenuating effects on the retinoid receptor specificity of heteroarotinoids.

Experimental Section

General. The NMR spectra were obtained on a Varian 300 MHz unit operating at 299.9 MHz for protons and 75.5 MHz for carbon-13. All samples were dissolved in $DCCl_3$ with TMS as the reference. All IR spectra were recorded as KBr pellets on a Perkin-Elmer 2000 FT-IR unit. Melting points were obtained on a Thomas-Hoover melting point apparatus and are uncorrected.

Synthesis and Properties of Heteroarotinoids 10 and 12−20. Compounds **13**,[5] **14**,[5] **15**,[4] **16**,[6] **17**,[5] **18**,[2] **19**,[5,27] and **20**,[5] were prepared as reported. The syntheses of compounds **10** and **12** are recorded herein.

1-(2,3-Dihydro-3,3-dimethyl-5-benzofuranyl)ethanone (7). A freshly prepared solution of the Grignard reagent from 5-bromo-2,3-dihydro-3,3-dimethylbenzofuran[5] [5.20 g, (22.9 mmol) and 1.7 g (70 mmol) of Mg turnings in dry THF (30 mL)] was added dropwise to a chilled (−40 to −45 °C), stirred solution of acetyl chloride (17 g, 0.22 mmol, 16 mL) in THF under N_2 and over 1 h. The resulting solution was allowed to warm to approximately −23 °C (∼20 min), and then stirring was continued for 2 h. Warming of the solution to about 0 °C was allowed over 40 min, and this solution was permitted to come to ambient temperature after which stirring was continued for 0.5 h. Quenching of the mixture was completed with water, and the aqueous layer was extracted (ether) repeatedly. The combined extracts were washed with 1 N NaOH and then with saturated brine. After drying ($MgSO_4$), evaporation of the solvent gave an oil which was vacuum distilled to remove low boiling fractions (<38 °C/0.12 mm). The residual oil was dissolved in a minimum of hexanes and chromatographed on silica gel with hexanes:ether (9:1 followed by 3:1). Crystallization of the oil was accomplished by repeated warming and chilling of a solution in a minimum of hexanes. The crystals were washed with chilled (−78 °C) hexanes and dried over P_2O_5 (room temperature, RT) which led to 2.25 g (51.6%) of **7** as very light tan crystals, mp 36.8−37.9 °C. Recrystallizations from chilled (−10 to − 15 °C) hexanes, followed by seeding, gave colorless crystals; mp 39−39.9 °C: IR (KBr) 1679 cm^{-1} (C=O); ^1H NMR ($DCCl_3$) δ 1.36 [s, 6 H, $C(CH_3)_2$], 2.55 [s, 3 H, $C(O)CH_3$], 4.33 [s, 2 H, OCH_2], 6.81 [d, 1 H, Ar−H], 7.77−7.86 [m, 2 H, Ar−H]; ^{13}C NMR ($DCCl_3$) ppm 26.4 [$C(O)CH_3$], 27.6 [$C(CH_3)_2$], 41.4 [$C(CH_3)_2$], 85.4 [OCH_2], 109.2 [C(7)], 163.6 [C(7a)], 122.9, 130.5, 131.0, 137.4 [Ar−C], 196.6 [C=O)]. Anal. calcd for $C_{12}H_{14}O_2$: C, 75.76; H, 7.42. Found: C, 75.72; H, 7.23. MS *m*/*z* ($M^{+\bullet}$) 190.0994; Found: 190.0998.

2-(2,3-Dihdro-3,3-dimethyl-5-benzofuranyl)-3-buten-2-ol (8). To freshly prepared solution of H_2C=CHMgBr [1.85 g (17.3 mmol) of H_2C=CHBr and 0.38 g (15.6 mmol) of Mg turnings in dry THF (10 mL)] was added, with stirring and under N_2, ketone **8** (1.00 g, 5.26 mmol) in dry THF (5 mL). The mixture was held at reflux (2 h) and at RT (2 h). The chilled (0 °C) mixture was diluted with ether and quenched with saturated NH_4Cl (pH ∼ 6−7). Addition of ether caused two layers to form, and the aqueous layer was repeatedly extracted (ether). The extracts were washed with $NaHCO_3$ (5%) and saturated brine and then dried (Na_2SO_4). Filtration of the organic layer followed by evaporation gave alcohol **8** as an oil (1.14 g, 99%) which was used without further purification. IR (neat) 3650−3150 (O−H) cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.34 [s, 6 H, $C(CH_3)_2$], 1.64 [s, 3 H, $C(OH)CH_3$], 1.94 [bs, 1 H, OH], 4.23 [s, 2 H, OCH_2], 5.13 [dd, J = 10.7 Hz, 1 H, HC=C(*H*)H], 5.30 [dd, J = 17.2 Hz, J = 1.0 Hz, 1 H, HC=CH(*H*)], 6.16 [dd, J = 17.2 Hz, J = 10.7 Hz, 1 H, *H*C=CH_2], 6.73 [d, J = 8.3 Hz, 1 H, H(7)], 7.19 [dd, J = 8.3 Hz, J = 2.0 Hz, 1 H, H(6)], 7.24 [d, J = 2.0 Hz, 1 H, H(4)].

[3-(2,3-Dihydro-3,3-dimethyl-5-benzofuranyl)-2-buten-1-yl]triphenylphosphonium Bromide (9). A solution of alcohol **8** (0.80 g, 3.66 mmol) in H_3COH (5 mL) was added dropwise to a stirred mixture of $Ph_3P{\cdot}HBr$ (1.25 g, 3.64 mmol) in H_3COH (5 mL) under N_2. The mixture became a solution and was stirred at RT for 20 h. Concentration gave a thick oil which was taken up with a minimum of H_3COH. The addition of cold ether caused crude salt **9** to precipitate. Purification of **9** was achieved by dissolving it in a minimum of H_3COH and allowing dry, cold ether to diffuse into the solution in a chamber. The crystals were filtered, washed (ether), and dried; 1.06 g (54%). Additional salt slowly precipitated from the mother liquors and was washed (ether) and dried. The solid was used without further purification. The total weight of purified **9** was 1.21 g (61%); mp 251.0−252.5 °C. IR (KBr) 3043 (Ar−H) cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.31 [s, 6 H, $C(CH_3)_2$], 1.59 [dd, $^5J_{PH}$ = 4.3 Hz, J_{HH} = 1 Hz, 3 H, HC=C(CH_3)], 4.21 [s, 2 H, OCH_2], 4.86 [dd, $^2J_{HP}$ = 15.1 Hz, J_{HH} = 7.8 Hz, 2 H, H_2C−P], 5.56 [m, 1 H, *H*C−CCH_3], 6.67 [d, J = 8.3 Hz, 1 H, H(7)], 6.91 [dd, J = 8.3 Hz, J = 1.8 Hz, 1 H, H(6)], 6.95 [d, J = 1.8 Hz, 1 H, H(4)], 7.66−7.96[m, 15 H, $P(C_6H_5)_3$].

(2*E*,4*E*,6*E*)-7-(2,3-Dihydro-3,3-dimethyl-5-benzofuranyl)-3-methyl-2,4,6-octatrienoic Acid (10). To a suspension of **9** (1.5 g, 2.76 mmol) in dry ether (20 mL) under N_2 was added a solution of *n*-butyllithium (1.6 M, 3.0 mmol, 1.9 mL) in hexanes in the dark. Stirring was continued at RT (15 min), and then the solution was cooled to −78 °C (∼15 min). A solution of (*E*)-OHC−$C(CH_3)$=$CHCO_2Et$ (1.22 g, 7.7 mmol) in dry ether (5 mL) was added slowly over 2 min. The cooling bath was removed, and stirring was continued for 46 h. Hexanes:ether (3:1) were added, and the mixture was stirred for 15 min and filtered. The solid was taken up in hexanes:ether (1:1), and this extract was filtered and added to the original filtrate. Evaporation of the solvent gave an oil which was chromatographed over silica gel (hexanes:ether, 20:1). An oil (0.46 g, 51%) was obtained which was treated with 35% KOH (1.5 mL) in absolute ethanol at reflux, under N_2 for 1 h. After cooling to RT, the mixture was treated with water (10 mL) and ethyl acetate (35 mL) and then quenched with acetic acid:water (1:1, 2 mL). The aqueous layer was extracted (ethyl acetate), and the combined organic solutions were dried (Na_2SO_4), filtered, and evaporated to a yellow solid. Recrystallization (absolute ethanol) and drying of the solid gave **10** (186 mg, 23% from the salt **9**); mp 204.0−205.2 °C. IR 3250−2300 (CO_2H), 1674 (C=O) cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.37 [s, 6 H, H(8,9)], 2.25 [d, J = 1 Hz, 3 H, H(11)], 2.40 [d, J = 1 Hz, 3 H, H(16)], 4.27 [s, 2 H, H(2)], 5.83 [bs, 1 H, H(17)], 6.40 [d, J = 14.9 Hz, 1 H, H(14)], 6.54 [d, J = 11 Hz, 1 H, H (12)], 6.77 [d, J = 8.3 Hz, 1 H, H(7)], 7.28 [dd, J = 8.3 Hz, J = 8.3 Hz, J = 2 Hz, 1 H, H(6)]. Anal. calcd for $C_{19}H_{22}O_3$: C, 76.48; H, 7.43. Found: C, 76.07; H, 7.55.

Methyl (*E*)-*p*-[4,4-Dimethylthiochroman-6-yl)propenyl]-benzoate (12). A solution of *n*-butyllithium (1.55 *M*, 2.01 mmol, 1.3 mL) in hexanes was added dropwise to a solution of [1-(4,4-dimethylthiochroman-6-yl)ethyl]triphenylphosphonium bromide[6] (1.1 g, 2.01 mmol) in anhydrous ether (90 mL, dried over sodium). After the addition was complete (the color changed from white to brick red), stirring was continued for 20 min at RT. The resulting solution was cooled to −78 °C, and a solution of methyl *p*-formylbenzoate (1.54 g, 9.4 mmol) in dry ether (50 mL) was added dropwise (the color changed from brick red to a light buff). The solution was then stirred for about 10 min, and the cooling bath was removed. After the mixture had reached RT, it was stirred for 48 h. The mixture was then filtered, and the residue was washed with anhydrous ether. The filtrate was then evaporated to a light yellow oil, which, upon refrigeration for 3 days, gave a light yellow solid. Chromatography of the solid taken up with a minimum of $HCCl_3$ was achieved over silica gel with hexane:ethyl acetate (1:5). Evaporation of the solution gave a light

yellow oil which solidified upon refrigeration. Recrystallization of the solid (95% ethanol) gave a white crystalline material (0.26 g, 37%); mp 124−125 °C. Spectral analysis gave the following results: IR (KBr)1715 (C−O) cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.37 [s, 6 H, $(CH_3)_2$], 1.99 [t, 2 H, H (3)], 2.27 [s, 3 H, =C−CH_3], 3.05 [t, 2 H, H(2)], 3.92 [s, 3 H, OCH_3], 6.79 [s, 1 H, C=CH (13)], 7.07 [m, 7 H, Ar−H]. Anal. calcd for $C_{22}H_{24}O_2S$: C, 74.96; H, 6.60; S, 9.09. Found: C, 74.85; H, 6.86; S, 9.05.

Biology. Receptor cotransfection assays were performed in CV-1 cells using automation in 96-well plates as previously described.[9] Briefly, RARα, RARβ, RARγ, or RXRα expression plasmid vectors were cotransfected by the calcium phosphate precipitation with a reporter luciferase (LUC) plasmid, pRS-β galactosidase plasmid as an internal control, and carrier plasmid pGEM. A basal reporter plasmid, MTV-LUC, containing two copies of the TRE-palindromic response element MTV-TREp-LUC was used in all transfections for RAR receptors. The reporter plasmid CRBPII-tk-LUC containing the RXRE from CRBPII was used with the RXRα transfections. Transfected cells were incubated for 40 h with or without various concentrations of retinoids. Cell extracts were prepared and assayed for luciferase and β-galactosidase as described by Berger et al.[28] All determinations were performed in triplicate and normalized for transfection efficiency by using β-galactosidase as a control. Retinoid activity was normalized relative to that of 9-*cis*-retinoic acid and is expressed as potency (EC_{50}), which is the concentration of retinoid required to produce 50% of the maximal observed response. The efficacy is the maximal response relative to 9-*cis*-retinoic acid.

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