Heteroarotinoids Inhibit Head and Neck Cancer Cell Lines in Vitro and in Vivo Through Both RAR and RXR Retinoic Acid Receptors

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A class of less toxic retinoids, called heteroarotinoids, was evaluated for their molecular mechanism of growth inhibition of two head and neck squamous cell carcinoma (HNSCC) cell lines SCC-2 and SCC-38. A series of 14 heteroarotinoids were screened for growth inhibition activity in vitro. The two most active compounds, one that contained an oxygen heteroatom (**6**) and the other a sulfur heteroatom (**16**), were evaluated in a xenograph model of tumor establishment in nude mice. Five days after subcutaneous injection of 10^7 SCC-38 cells, groups of 5 *nu/nu* mice were gavaged daily (5 days/week for 4 weeks) with 20 mg/kg/day of *all-trans*-retinoic acid (*t*-RA, **1**), 10 mg/kg/day of **6**, 10 mg/kg/day of **16**, or sesame oil. After a few days, the dose of *t*-RA (**1**) was decreased to 10 mg/kg/day to alleviate the side effects of eczema and bone fracture. No significant toxic effects were observed in the heteroarotinoid groups. All three retinoids caused a statistically significant reduction in tumor size as determined by the Student *t*-test ($P < 0.05$). Complete tumor regression was noted in 3 of 5 mice treated with *t*-RA (**1**), 4 of 5 mice treated with **16**, 1 of 5 mice treated with **6**, and 1 of 5 mice treated with sesame oil. Reverse transcriptase polymerase chain reaction (RT-PCR) was used to determine that the expression levels of RARα, RXRα, and RXRβ were similar in the two cell lines, while RARβ expression was higher in SCC-2 over SCC-38, and RARγ expression was higher in SCC-38 over SCC-2. Receptor cotransfection assays in CV-1 cells demonstrated that **16** was a potent activator of both RAR and RXR receptors, while **6** was selective for the RXR receptors. Transient cotransfection assays in CV-1 cells using an AP-1 responsive reporter plasmid demonstrated that *t*-RA (**1**), **6**, and **16** each inhibited AP-1-driven transcription in this cell line. In conclusion, the growth inhibition activity of the RXR-selective **6** and the more potent growth inhibition activity of the RAR/RXR pan-agonist **16** implicate both RARs and RXRs in the molecular mechanism of retinoid growth inhibition. Moreover, the chemoprevention activity and the lack of toxicity of heteroarotinoids demonstrate their clinical potential in head and neck cancer chemoprevention.

Introduction

Retinoids have been known to elicit useful pharmacological responses in a variety of disorders.[1] Activities of endogenous retinoids such as *trans*-retinoic acid (*t*-RA, **1**), 9-*cis*-retinoic acid (9-*c*-RA, **2**), and 13-*cis*-retinoic acid (13-*c*-RA, **3**) are well-known in cancer therapy, although all have some toxic characteristics.[2] The synthetic retinoid 4-(hydroxyphenyl)retinamide (**4**, 4-HPR), currently under clinical investigation, is also known to possess some undesirable properties such as night blindness, although it appears to be dose-dependent and reversible.[3]

Certain heteroarotinoids have demonstrated anticancer activity as well as low toxicity.[4−8] We now report the growth inhibitory activity of a variety of heteroarotinoids **5**−**19** (Chart 1) against head and neck squamous cell carcinoma (HNSCC) cell lines, SCC-2 and SCC-38. The heteroarotinoids possess a range of flexibility in terms of the linking groups attached to the aryl ring and, in most cases, between two aryl rings. It was reasoned that such flexibility might better allow accommodation of the molecule in the receptor site and

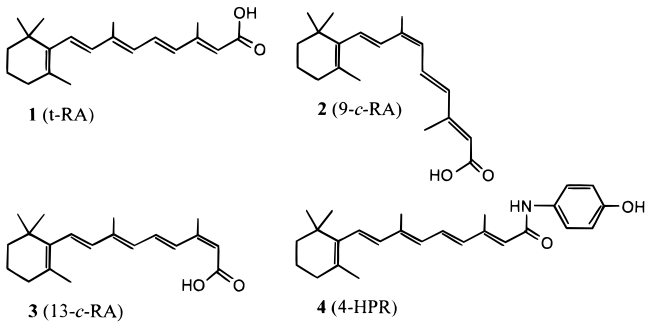

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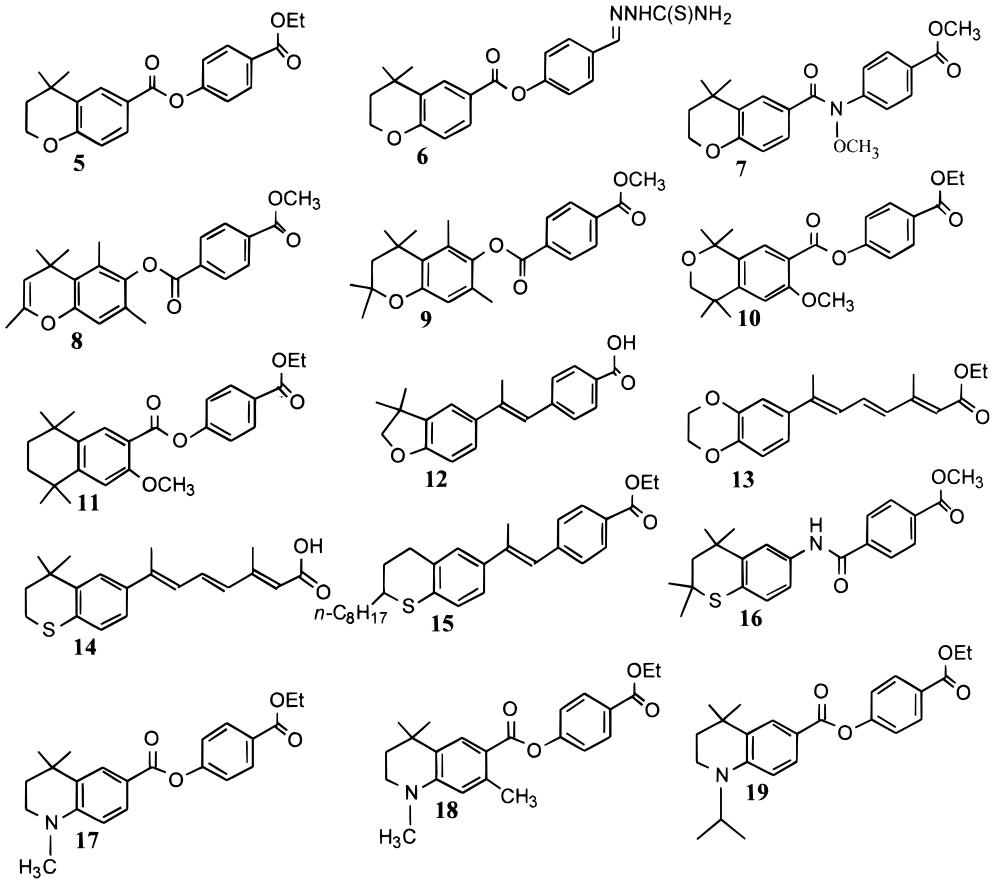
Chart 1



therefore lead to an activated complex of ligand−receptor. The area of ligand−receptor binding has been reviewed.[9]

Head and neck cancers often arise from a field of epithelial injury caused by carcinogens in tobacco smoke which, in turn, leads to multiple premalignant lesions and the risk of primary and second primary tumor formation.[10,11] Retinoids have been shown to reverse oral premalignant lesions and prevent second primaries in patients treated for head and neck cancers. The use of retinoids for the prevention and treatment of head and neck cancers was inspired by the observations that vitamin A deficiency caused keratinizing squamous metaplasia in mucociliary epithelium and that replenishment of retinoids restored normal epithelial differentiation.[12,13] Leukoplakia and erythroplakia are premalignant head and neck mucosal lesions. Several studies have attempted to use retinoids to prevent or reverse these lesions back to normal mucosa in high-risk patients. In a low-dose experiment that showed minimal toxicity to the patients, the natural retinoid 13-*c*-RA (**3**) was found to be superior to β-carotene in reducing the rate of oral leukoplakia progression.[14] The synthetic retinoid 4-HPR (**4**) has been shown to decrease relapses and new localizations of surgically treated oral leukoplakia from 30% to 6%.[15]

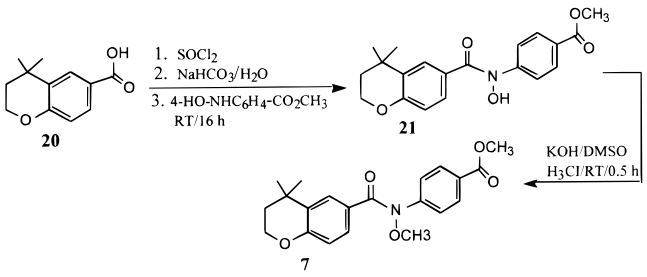
Many head and neck epithelial malignancies are multifocal, and the development of a second primary tumor either during treatment or after successful treatment for a primary tumor occurs at a rate of 4−7%/year.[16] A population of these patients had significant reductions in development of second primary tumors when treated with 13-*c*-RA (**3**) for a median follow-up of 54 months.[17] Despite these encouraging results, a large number of relapses have been detected upon cessation of certain agents, and there have been numerous side effects, especially at high doses. These side effects include mucocutaneous dryness, dermatitis, conjunctivitis, photodermatosis, impaired night vision, hypertriglyceridemia, and transient increases in serum liver function enzymes. Efforts to prevent relapses and to reduce toxicity have included co-treatment with α-tocopherol and the development of synthetic retinoids.[7,18]

Prior to clinical application, the chemoprevention activities of retinoids are usually evaluated against HNSCC cell lines in vitro and in animal models in vivo. Although vitamin A is the least toxic of the natural retinoids, it is also the weakest inhibitor of growth in HNSCC cell lines in vitro and in xenograph growth in vivo, followed by 13-*c*-RA (**3**).[19−24] The reduced toxicity of certain heteroarotinoids[7] prompted an evaluation in the present study to determine if such heterocycles inhibited the growth of HNSCC cell lines in vitro and in vivo and to explore the molecular mechanism of action of such agents.

Results and Discussion

Chemistry. The discussion of the chemistry is in an order with respect to various properties of the selected heteroarotinoids. Known activities of **5**,[7] **6**,[7] **12**,[6] **14**,[4] and **15**[7] in certain assays prompted us to make a change to a somewhat different flexible linking group as in **7**. Although some amide linking groups have been exam-

Scheme 1



ined for useful activity[25] and even for RARα selectivity,[26] no record of the use of a C(O)−N−methoxy linker could be found in the literature. The excess bulk of this group was considered as altering the rotational barrier of the C−N bond and thereby possibly influencing receptor binding and activation.

Generation of the acid chloride from known **20**[8] and subsequent treatment with the appropriate hydroxylamine as shown gave **21** (Scheme 1). *O*-Methylation proceeded under mild conditions to yield **7** (48%).

Creating and assessing ester **8** for useful activity was considered a test of the influence on activity by flanking methyl groups of the aryl ring with respect to freedom of rotation of the internal ester linkage. In addition, it is known[5,7] that alkyl groups adjacent to the heteroatom have a positive influence on biological activity. Thus, ester **8** possesses both such features. Excess methylmagnesium bromide added to known **22**[27] in boiling ether over 48 h led to lactol **23** (Scheme 2). Dehydration of **23** under standard conditions yielded ether **24**. Direct esterification of **24** gave new ester **8** (50%) which was purified by chromatography.

Surprisingly, when **22** was treated with the same excess of methyl Grignard reagent as used to obtain **23**, but in THF, ether **25** (76%) formed (Scheme 3). Perhaps the much longer reaction time was significant, but the solvent was found to be important as well. Esterification of **25** under the usual conditions produced the target ester ether **9** (52%). This heteroarotinoid **9** possessed the geminal methyl flanking group that enhanced activity in several assays in related systems which contained more rigid linking groups attached to the aryl ring.[5] Consequently, an assessment could be made of the alteration in activity between **8** and **9**.

Retaining the flexible ester linkage between the two aryl rings was desirable in **10**. In part, the rationale for **10** was based on the strong activity of (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB), although it is very toxic.[7] The incorporation of an oxygen atom into system **10** might reduce toxicity sufficiently to be tolerable and yet retain useful activity. Dimethylation of **26** as shown was facile and gave **27** in high yield (84%) (Scheme 4). Base-catalyzed hydrolysis of **27** led to acid **28** (69%), a reported compound[28] but not previously well-characterized. Reduction of **28** to **29** was straightforward, and alcohol **29** was cyclized with the incorporation of an acetone unit to yield ether **30** (75%). Acetylation of **30** proved more difficult than anticipated, but the use of H_3CNO_2 with $AlCl_3$ and AcCl was successful and gave **31** (51%) *without* cleavage of the methoxy ether group. Oxidative cleavage of the acetyl group in **31** to give acid **32** gave only a modest yield (21%) due to side reactions. The use of a combination of LiOCl/Clorox was ultimately successful. Esterification of **32** required a long reaction time to obtain **10**, apparently due to the increased steric hindrance around the carbonyl group of **32**.

In view of the known toxicity of TTNPB,[7] the synthesis of the close analogue **11** (Scheme 5) would also serve as a model for comparison of activity with that of heteroarotinoid **10**. Acetylation of known[29] **33** also was demanding in terms of avoiding or minimizing cleavage of the ether linkage in **30**. Using the conditions outlined resulted in the generation of **34** (75%). As with the conversion of **31** to **32**, generation of acid **35** from ketone **34** was slow because of hindrance, although **35** was isolated in moderate yield (44%). Esterification of **35** to produce **11** (67%) required a long reaction time as anticipated.

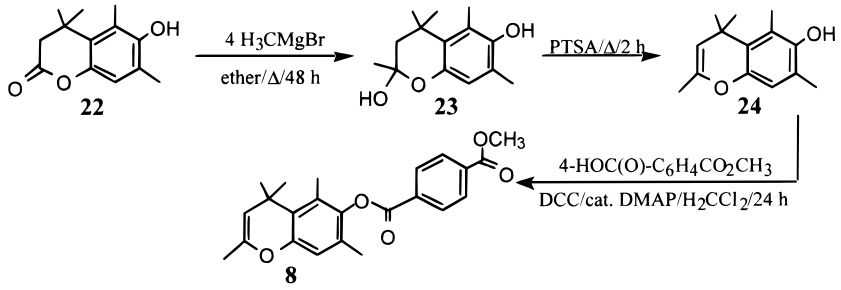
The unusual heteroarotinoid **13** was selected for inclusion in the study since it possessed a rigid long side chain and contained two heteroatoms in the fused ring unit. Other heteroarotinoids with long side chains had exhibited strong activity in several assays.[5,6] The influence of two heteroatoms could also be assessed. The addition of a nearly 3-fold excess of vinylmagnesium bromide to commercial **36** resulted in essentially a quantitative yield of slightly crude **37** (Scheme 6). Conversion of the vinyl alcohol **37** to the phosphonium salt **38** (85%) was effected by conditions previously developed.[5,7] Generation of the Wittig reagent of **38** as shown was followed by the addition of ethyl 3-methyl-4-oxocrotonate in a normal condensation to provide heteroarotinoid **13**, albeit in modest yield (28.8%).

The flexible heteroarotinoid **16** could be obtained via the conversion of **39** → **40** → **41** → **16** as illustrated (Scheme 7). The nitration of known[5] **39** afforded the 6-isomer **40** in modest yield (27%) since other nitrated products appeared to have formed. Reduction of the nitro group in **40** to the amino function in **41** (50%) progressed reasonably well under mild conditions. *N*-Acylation of **41** with the acid chloride shown produced the target **16** (72%), again under quite mild conditions.

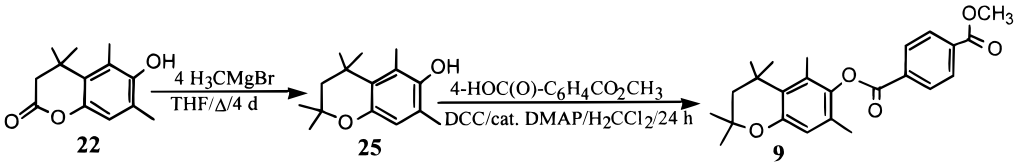
Biology. 1. In Vitro Growth Inhibition Screen. A series of retinoids were screened for growth inhibition activity in the SCC-2 and SCC-38 HNSCC cell lines. The activities of 14 heteroarotinoids containing a sulfur heteroatom (**14**−**16**), an oxygen heteroatom (**5**−**10, 12, 13**), or a nitrogen heteroatom (**17**−**19**), as well as a structurally related arotinoid (**11**) void of a heteroatom, were compared to that of 9-*c*-RA (**2**) (Table 1). Although most of the compounds inhibited growth, some of them stimulated growth. Three of the compounds with oxygen heteroatoms (**5**, **8**, **9**) had opposite effects in the two cell lines. The Student *t*-test was used to demonstrate that there were no statistically significant differences between the responses of the two cell lines to the entire set of retinoids ($P > 0.05$) or between the activities of groups with different types of heteroatoms ($P > 0.05$). The oxygen heteroarotinoid **6** and sulfur heteroarotinoid **16** were the most active in both cell lines and were therefore chosen for evaluation in a xenograft model.

2. Xenograft Model. The **6** and **16** compounds were evaluated for biological activity in a xenograft nude mouse model. Athymic *nu*/*nu* mice were injected subcutaneously with 10^7 SCC-2 or SCC-38 cells. The effects of retinoids in mice injected with SCC-2 cells could not

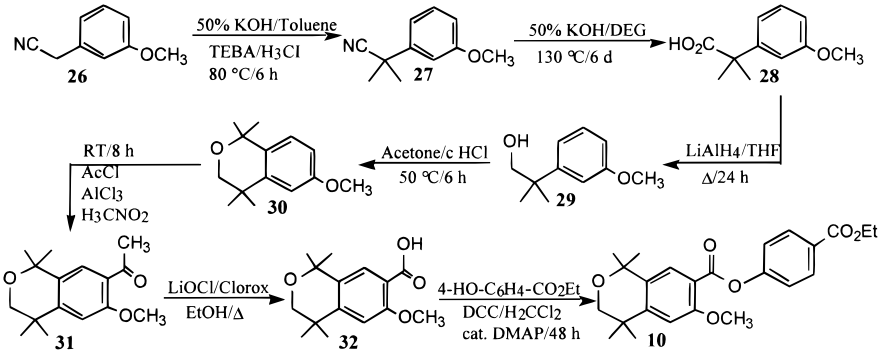
Scheme 2



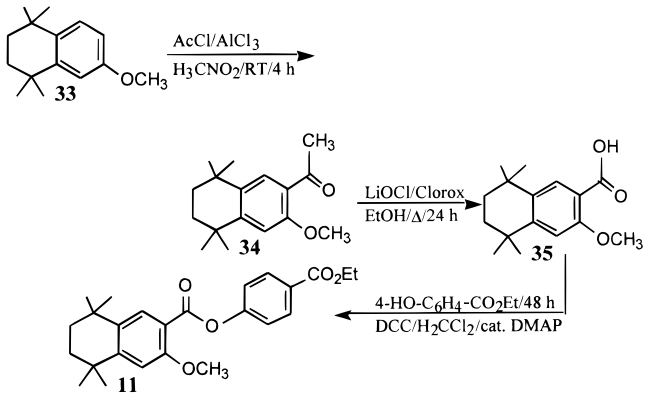
Scheme 3



Scheme 4



Scheme 5



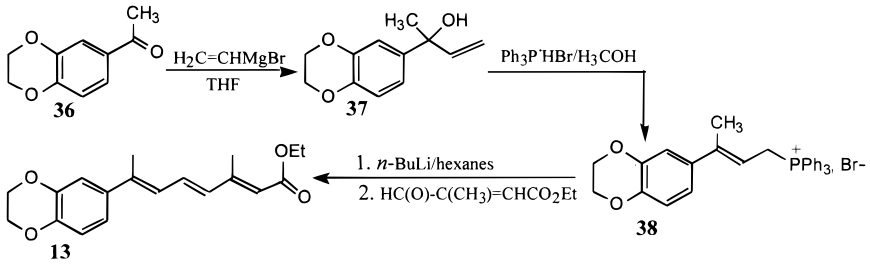
be evaluated because these mice developed very large tumors and died rapidly of disease (data not shown). Mice injected with SCC-38, however, developed stable tumors and survived the duration of the month-long experiment. Mice bearing SCC-38 xenografts were arbitrarily divided into 4 treatment groups of 5 mice each and were gavaged with 0.1 mL of sesame oil containing either 20 mg/kg/day of *t*-RA (**1**), 10 mg/kg/day of **6**, 10 mg/kg/day of **16**, or no drug control. After 1 week of treatment, the dose of *t*-RA (**1**) was decreased to 10 mg/kg/day due to excessive eczema and bone fractures in this group of mice. These toxicities were not evident in the other three treatment groups. There was no significant difference in the animal weights between the treatment groups (Figure 1, panel A).

Tumors developed from the SCC-38 cell line underwent a reduction in volume during days 9−14, followed by a stabilization of tumor size (Figure 1, panels B−D). The tumors in the groups treated with *t*-RA (**1**) and **16** stabilized starting on days 12 and 14, respectively, at sizes that were significantly smaller than the tumors in the control group ($P < 0.05$, *t*-test), which were also stable during this period. The sizes of the tumors in the group treated with **6** were not significantly smaller than the tumors of the control group until day 26 ($P < 0.05$, *t*-test). The last day of tumor measurements demonstrated growth in the control group compared to continued stabilization or further reduction in the treatment groups. The average tumor volumes of the four treatment groups were significantly different from each other after day 23 as determined by ANOVA ($P < 0.05$).

Complete tumor regression was observed in 3 of 5 mice treated with *t*-RA (**1**), 4 of 5 mice treated with **16**, and 1 of 5 mice treated with **6**. In the control group, 1 of 5 sesame oil-treated mice had complete tumor regression.

3. Receptor Expression. Retinoids regulate gene expression through two classes of nuclear retinoic acid receptors, RAR and RXR, each of which have three isoforms labeled α, *β*, and *γ*.[9] The expression patterns of retinoic acid receptors in SCC-2 and SCC-38 were evaluated by reverse transcriptase polymerase chain reaction (RT-PCR). Both cell lines expressed RARα, RXRα, and RXR*β* at similar levels (Figure 2). The intensities of the *β*actin control bands for each cell line were similar, but the intensities of the RAR*β* and RAR*γ*

Scheme 6



Scheme 7

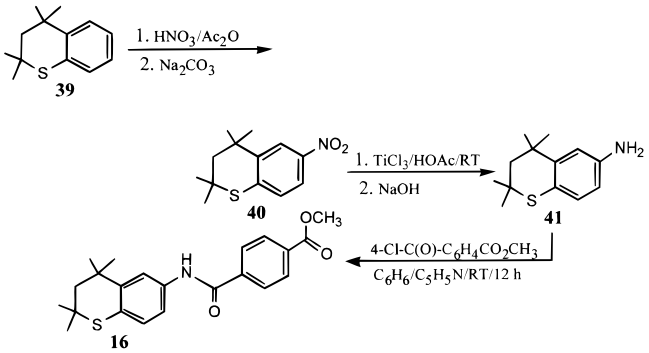


Table 1. Percent in Vitro Growth Inhibition of Two HNSCC Cell Lines, SCC-2 and SCC-38, by a 3-Day Exposure to 10 μM Heteroarotinoids and 9-*c*-RA (**2**)

atom	compd	SCC-2	SCC-38
O	**5**	−6	13
O	**6**	19	18
O	**7**	3	17
O	**8**	15	−8
O	**9**	−1	9
O	**10**	52	6
O	**12**	1	1
O	**13**	−33	−2
S	**14**	2	1
S	**15**	10	17
S	**16**	64	37
N	**17**	12	13
N	**18**	9	7
N	**19**	5	15
	11	15	12
	9-*c*-RA (**2**)	13	10

bands for each cell line varied, indicating that SCC-2 expressed higher levels of RARβ in comparison to SCC-38 and that SCC-38 expressed higher levels of RARγ in comparison to SCC-2.

4. Nuclear Retinoid Receptor Activation. While *t*-RA (**1**) and arotinoid compounds only activate the RAR receptors, 9-*c*-RA (**2**) and some heteroarotinoids activate both RAR and RXR receptors.[9] To determine the specificity of retinoic acid receptor activation by **6** and **16** cotransfection assays using the RARβ RARE-tk-CAT reporter plasmid were performed. All six nuclear receptors were activated by **6** and **16** in a dose-responsive manner. The concentration that induced half-maximal activity (EC_{50} value) was derived as a measure of receptor potency, and the maximal activity relative to 9-*c*-RA (**2**) was derived as a measure of efficacy for each receptor (Table 2). Heteroarotinoid **16** was a potent activator of all six receptors, while **6** was selective for the RXR receptors. Although the efficacy of RAR activation by **6** ranged from 24−58% of the activation by 9-*c*-RA (**2**), the greater than 10 000 nM potencies demonstrate that the concentrations needed to achieve this activation were above pharmacologically obtainable levels.

5. Anti-AP-1 Activity. The ligand-bound retinoic acid receptors have been shown to repress transcriptional activation by AP-1 transcription factors.[30,31] This anti-AP-1 activity has been implicated in the mechanism of growth inhibition.[32,33] The ability of *t*-RA (**1**), 9-*c*-RA (**2**), **6**, and **16** to repress AP-1-driven transcription was evaluated using transient transfection assays of an AP-1-driven reporter plasmid into SCC-2 cultures (Figure 3). All the retinoids repressed AP-1 activity, and **16** exhibited the greatest repression. Similar experiments using SCC-38 cultures could not be performed due to the poor transfection efficiency of this line.

Conclusions

In this report, the potential of certain heteroarotinoids for chemoprevention of head and neck cancers was confirmed by growth inhibition of HNSCC cell lines in vitro and prevention of tumor establishment in a xenograph nude mouse model in vivo. The SCC-38 xenograph tumors in this study exhibited a reduction during days 9−14, followed by a stabilization of tumor size (Figure 1). The curves in all four groups were remarkably similar for days 9−14, suggesting that this initial regression in size was due to factors intrinsic to the xenograph model such as absorption of diluent and nonviable tumor cells and resolution of the host's inflammatory response to tumor injection. The last tumor measurement in the control group, however, demonstrated significant growth compared to continued stabilization or further reduction in the treatment groups. This implies that the model is reflective of tumor establishment rather than tumor growth. Establishment of tumor implants appears to have occurred over the initial 28 days following injection, while growth of the established tumors did not occur until after day 28. The efficacy of the two heteroarotinoids in prevention of the establishment of SCC-38 xenograph tumors is indicated by the significant decrease in size of the tumors in the groups treated with **6** and **16**. The complete regression of 4 out of 5 xenograph tumors in the group treated with **16**, in comparison to 1 out of 5 in the control group, is a more convincing demonstration of heteroarotinoid chemoprevention activity.

The molecular mechanism of this growth inhibition most likely involves the regulation of gene expression through the nuclear retinoic acid receptors. In support of this hypothesis, the use of *t*-RA (**1**) to treat squamous cell carcinoma HNSCC cell lines resulted in decreased expression of two genes that are associated with increased proliferation and that are commonly overex-

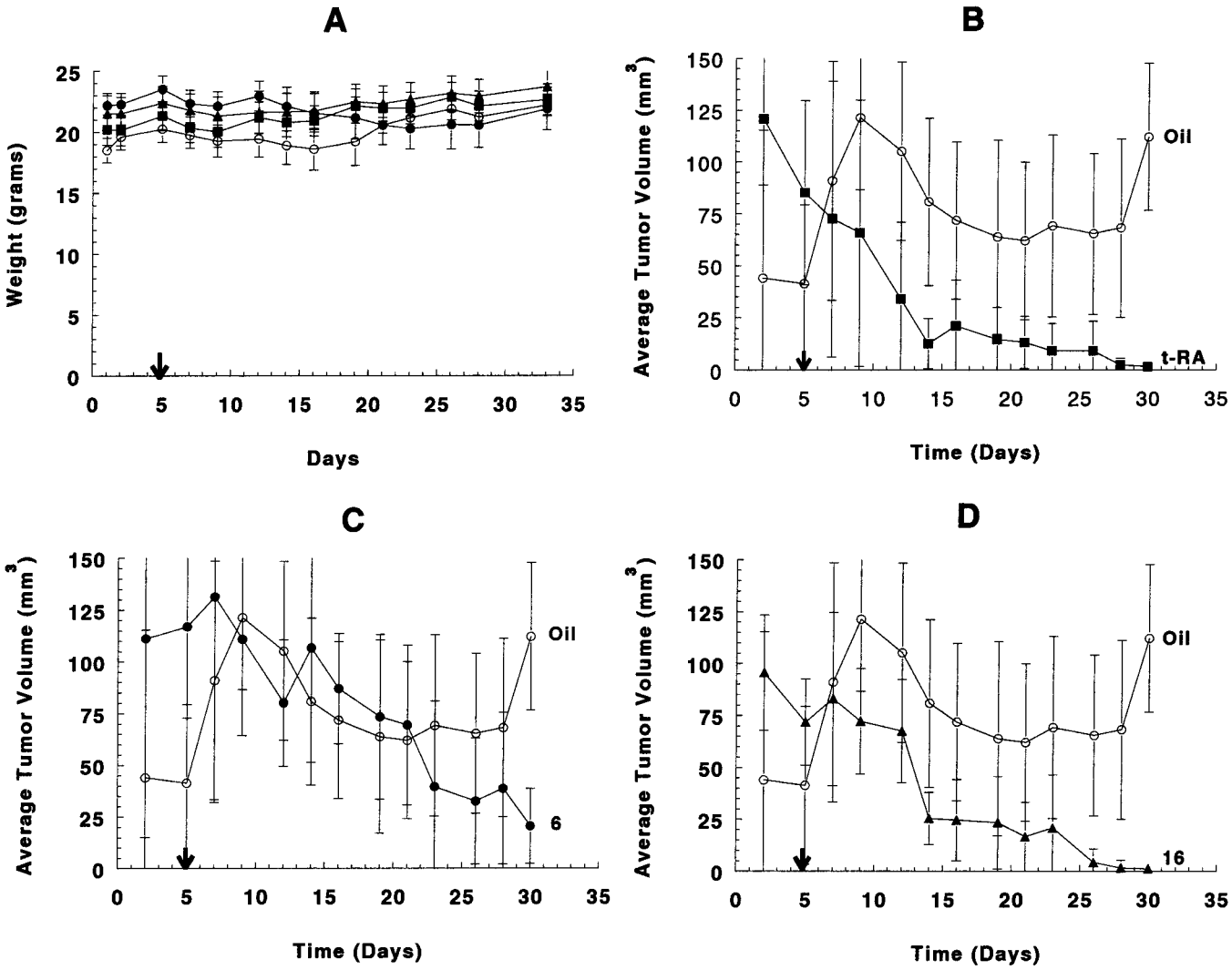


Figure 1. Xenograft model of retinoid-induced tumor shrinkage. An amount of 10^7 SCC-38 cells was injected into the flanks of 18 nude mice. Five days later, 10 mg/kg of *t*-RA (**1**) (■), **6** (●), **16** (▲), or sesame oil (○) was administered by gavage for 5 consecutive days/week. Each mouse was weighed 3 times/week, and the average weights for each group are shown in panel A. Tumor volumes were measured with calipers. The data in panels B−D represent the average and standard deviations of tumor sizes of 6 mice/group treated with *t*-RA (**1**), **6**, or **16**, respectively. Retinoid treatment caused a statistically significant decrease in tumor size starting at day 12 for *t*-RA ($P = 0.025$), day 26 for **6** ($P = 0.051$), and day 14 for **16** ($P = 0.016$) as determined by the Student T-test.

pressed in head and neck cancers, namely the transforming growth factor α (TGF-α) and the epidermal growth factor receptor (EGF-R).[34] The growth inhibition activities of compounds, such as *t*-RA (**1**), that activate all RAR receptors are not likely to be strongly affected by alterations in the pattern of receptor expression. In agreement with this suggestion, a study using a panel of six HNSCC cell lines revealed that receptor expression patterns were unrelated to a response to *t*-RA (**1**) treatment.[21] In contrast, the activities of receptor-selective retinoids can be affected by the receptor expression patterns in head and neck lesions. An investigation of premalignant, dysplastic, and malignant head and neck cancers found that RARβ expression was suppressed in more than 50% of all lesions.[23] However, RARγ expression was not suppressed and has been implicated in squamous differentiation and *t*-RA response in HNSCC.[35,36] The role of RARγ in the mechanism of retinoid growth inhibition in HNSCC cell lines has been illustrated by the use of sense and antisense RARγ expression in the SqCC/Y1 line.[36] While *t*-RA (**1**) treatment and sense RARγ expression caused growth inhibition, AP-1 repression and decreased expression of EGF-R and of squamous differentiation markers, anti-sense RARγ, antagonized these activities. Therefore, the potencies of RARβ and RARγ activation by **16** (EC_{50}'s of 1004 and 152 nM, respectively) are amenable to use against head and neck lesions that have decreased expression of RARβ and consistent expression of functional RARγ.

Both SCC-2 and SCC-38 cell lines evaluated in this study expressed all of the human RAR and RXR nuclear retinoic acid receptors and are therefore susceptible to all retinoic acid receptor-selective compounds. The potent growth inhibition activity of **6**, which is selective for the RXR receptors, suggests that RXR activation is sufficient for growth inhibition of SCC-2 and SCC-38. The greater activity of **16**, which is an RAR/RXR pan-agonist, indicates that RARs also contribute to the mechanism of growth inhibition by heteroarotinoids. Additive and synergistic activity of RAR- and RXR-selective retinoids has been noted in other cell lines in

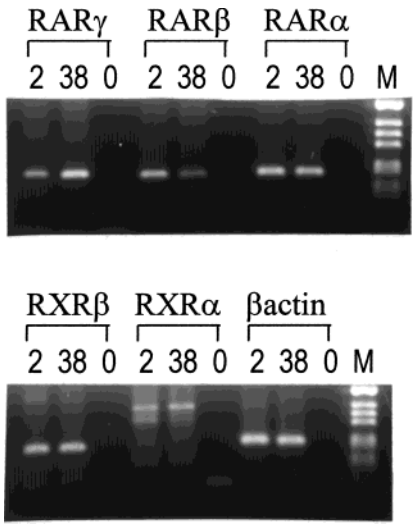


Figure 2. RAR expression profile of SCC-2 and SCC-38. RT-PCR reaction products, obtained with primers specific for each receptor, were electrophoresed through 1.5% agarose gels containing ethidium bromide and photographed. The expected size of the specific bands for RARα, RARβ, RARγ, and RXRβ is approximately 200 base pairs. The expected size for RXRα is 400 base pairs. The lanes contain RT-PCR reaction products from SCC-2 (2), SCC-38 (38), or no RNA control (0) and molecular weight markers (M) (1 kb DNA ladder, GibcoBRL, Gaithersburg, MD; the top band corresponds to 505 bp's followed by 396, 344, 288, 220, 201, 154, 134, and 75).

vitro.[37] The inhibition of HNSCC xenograph tumor growth by an RAR-selective retinoid, observed by others,[38] and by RXR-selective retinoid **6**, in this study, demonstrates that either RAR or RXR activation is sufficient for growth inhibition. The more potent activity of RAR/RXR pan-agonist **16** suggests that activation of both RAR and RXR receptors is also complimentary in vivo.

Since all of the retinoids used in the animal model repressed AP-1-driven transcription to similar extents, the contribution of anti-AP-1 activity to the mechanism of growth inhibition cannot be ruled out. Although the most biologically active heteroarotinoid **16** exhibited the greatest repression of AP-1 activity, it was also the most active RARE transactivator and, therefore, cannot be used to differentiate the two retinoid activities. It is most likely, however, that both anti-AP-1 and RARE transactivation activities are involved in the mechanism of retinoid growth inhibition to different degrees, depending upon the inner cellular environment. A series of heteroarotinoids are currently being investigated for selected anti-AP-1 activity in the absence of RARE transactivation activity. These types of selective compounds can be used to evaluate the role of AP-1 repression in the mechanism of growth inhibition.

In conclusion, the growth inhibitory activity of RXR-selective **6** and the more potent growth inhibition activity of RAR/RXR pan-agonist **16** implicate both RARs and RXRs in the molecular mechanism of retinoid growth inhibition of HNSCC cell lines. Both agents exhibit anti-AP1 activity that may contribute to the mechanism of action. Moreover, the chemoprevention activity and lack of toxicity of heteroarotinoids demonstrate their clinical potential in head and neck cancer chemoprevention.

Experimental Section

Chemical Methods. General. IR spectra were recorded on a Perkin-Elmer 2000 FT-IR as films or as KBr pellets. GC−MS data were obtained from a HP G1800A GCD System GC electron ionization detector. FAB MS experiments were performed on a VG ZAB-2SE HRMS unit, while EI MS experiments were accomplished with an HP GC-MS ENGINE model 5989B unit. All ^{1}H and ^{13}C NMR spectra were taken on a Varian Inova 400 MHz or Varian XL-400 MHz BB spectrometer operating at 399.99 and 100.0 Hz, respectively, and signals were referenced to TMS. Melting points were determined with a Thomas-Hoover melting point apparatus and were uncorrected. Syntheses were executed, unless otherwise indicated, under an atmosphere of N_2. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN, or by Atlantic Microlab, Inc., Norcross, GA. All chromatography was performed with the chromatotron unless otherwise noted and utilized silica gel, pF 254 containing gypsum (EM Science).

Heteroarotinoids **5**,[7] **6**,[7] **12**,[6] **14**,[6] **15**,[7] **17**,[39] **18**,[39] and **19**[39] were prepared by known methods. Although the intermediates and final products appeared to be relatively stable in light, precautions were taken to minimize exposure to any light source and to the atmosphere. In the purified state, all materials were stored in the cold and dark for indefinite periods without significant decomposition.

Biological Methods. Cell Lines. The SCC-2 cell line was derived from a patient with T1,N0 well-differentiated squamous cell carcinoma of the mandibular alveolus. The SCC-38 cell line was derived from a patient with a T2,N2a moderately well-differentiated squamous cell carcinoma of the base of the tongue. Cells were maintained in Eagles minimal essential media (MEM) containing Earle's salts and L-glutamine, supplemented with nonessential amino acids, antibiotic-antimycotic, sodium pyruvate, and 10% fetal bovine serum (FBS) in 95% air−5% CO_2. Only lots of FBS that had negligible quantities of retinoic acid as determined by HPLC ($<10^{-8}$ M) were used.

Retinoids. All heteroarotinoids (**5**−**10**, **12**−**19**), arotinoid **11**, *t*-RA (**1**; Sigma Chemical Co., St. Louis, MO), and 9-*c*-RA (**2**; Bio-Mol, Plymouth Meeting, PA) were dissolved in dimethyl sulfoxide (DMSO) for in vitro studies or highly refined sesame oil (Croda, Inc., Mill Hall, PA) for in vivo studies, were protected from light, and were stored at −70 °C. For the tissue culture studies, retinoids were added from 1000X stocks, achieving a final concentration of 0.01% DMSO in the culture medium. For the animal studies, the retinoids were dissolved at a concentration equal to the daily dose of 0.1 mL of sesame oil.

Methyl *N*-(4-Methoxyoxophenyl)-4,4-dimethyl-3,4-dihydro-2*H*-benzo[*b*]pyran-6-ylhydroxamate (7). A mixture of powdered KOH (0.022 g, 0.38 mmol) and DMSO (1 mL) was stirred for 5 min, and then ester **21** (0.090 g, 0.25 mmol) was added, followed by an immediate addition of H_3C-I (0.031 mL, 0.51 mmol) with stirring. After stirring for 0.5 h, the resulting mixture was poured into H_2O (10 mL). Extraction (H_2CCl_2, 3 × 10 mL) of the aqueous layer and combining the extracts gave a solution which was washed with water and brine. After drying ($MgSO_4$), the solution was evaporated to a colorless solid which was recrystallized (hexanes:EtOAc, 4:1) to give **7** (0.045 g, 48%): mp 104−106 °C; IR (KBr) 1722, 1670 (C=O) cm^{-1}; ^{1}H NMR ($DCCl_3$) δ 1.29 [s, 6 H, $(H_3C)_2$], 1.83 [t, 2 H, J = 5.6 Hz, CCH_3], 3.71 [s, 3 H, $NOCH_3$], 3.92 [s, 3 H, OCH_3], 4.23 [t, 2 H, J = 5.6 Hz, OCH_2], 6.77 [d, 1 H, Ar−H], 7.47 [dd, 1 H, Ar−H], 7.59 [dd, 2 H, Ar−H], 7.63 [d, 1 H, Ar−H], 8.04 [dd, 2 H, Ar−H]; ^{13}C NMR ($DCCl_3$) ppm 30.53 [Ar*C*CH_2], 30.74 [$(H_3C)_2$], 37.05 [Ar*C*CH_2], 51.15 [OCH_3], 62.17 [$NOCH_3$], 63.32 [ArOCH_2], 116.59−156.38 [Ar−C], 166.38 [C=O], 167.97 [C=O]. Anal. ($C_{21}H_{23}NO_5$) C, H, N.

Methyl 4-[(6-Hydroxy-2,4,4,5,7-pentamethyl-4*H*-benzo[*b*]pyran-6-yl)carbonyloxy]benzoate (8). To a cloudy mixture of the chromenol **24** (0.150 g, 0.688 mmol), methyl monoterephthalic acid (0.161 g, 0.894 mmol), and H_2CCl_2 (10 mL) were added slowly dicyclohexylcarbodiimide (0.425 g, 2.06 mmol) and a catalytic amount (7 mg) of DMAP. After the resulting cloudy solution was stirred (rt) for 24 h and filtered,

Table 2. Potency (EC_{50}, nM) and Efficacy (maximal response relative to 9-*c*-RA (**2**)) of Heteroarotinoids[a]

	potency (efficacy)					
	RAR			RXR		
compd	α	*β*	*γ*	α	*β*	*γ*
6	>10000 (24)	>10000 (58)	>10000 (38)	294 (35)	1897 (46)	42 (35)
16	225 (72)	1004 (73)	152 (86)	84 (80)	68 (49)	302 (43)

[a] The efficacy was derived by dividing the maximal activity induced by **6** or **16** by the maximal activity induced by 9-*c*-RA (**2**). All compounds exhibited maximal activity at a concentration of 10 μM. The maximal activity induced by 10 μM 9-*c*-RA (**2**) was 4.2-fold induction for RARα, 2.6-fold for RAR*β*, 2.4-fold for RAR*γ*, 3.8-fold for RXRα, 4.0-fold for RXR*β*, and 3.7-fold for RXR*γ*.

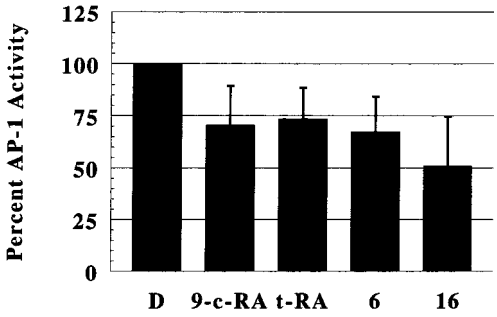


Figure 3. Repression of AP-1-driven transcription. SCC-2 cultures transiently cotransfected with an AP-1 responsive reporter plasmid (ColCAT) and a transfection control plasmid (pJATLac) were treated with 10 μM of the indicated retinoid. After 48 h of treatment, cell lysates were prepared and CAT expression was quantitated as a measure of AP-1 activity. The results are presented as the average and standard deviations of 4 determinations.

the filtrate was cooled (0 °C) overnight and filtered again. Evaporation of the solvent gave a heavy oil which was subjected to chromatography (H_2CCl_2). A white solid (0.130 g, 50%) **8** was obtained: mp 142−143 °C; IR (KBr) 1736 (C=O) cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.44 [s, 3 H, CH_3], 1.57 [s, 3 H, CH_3], 2.12 [s, 3 H, Ar−CH_3], 2.27 [s, 3 H, Ar−CH_3], 3.98 [s, 3 H, OCH_3], 4.39 [s, 1 H, C=CH], 6.72 [s, 1 H, Ar−H], 8.18 [dd, 2 H, Ar−H], 8.30 [dd, 2 H, Ar−H]; ^{13}C NMR ($DCCl_3$) ppm 14.88 [CH_3], 16.23 [CH_3], 18.78 [CH_3], 31.79 [$ArCCH_2$], 32.54 [$(H_3C)_2$], 52.51 [OCH_3], 109.00−148.51 [*C*=*C*] and [Ar−C], 163.83 [C=O], 166.13 [C=O], 168.07 [C=O]. Anal. ($C_{23}H_{24}O_5$) C, H.

Methyl 4-[(2,2,4,4,5,7-Hexamethyl-3,4-dihydro-2*H*-benzo[*b*]pyran-6-yl)carbonyloxy]benzoate (9). To a cloudy mixture of **25** (0.085 g, 0.366 mmol) and methyl monoterephthalic acid (0.115 g, 0.641 mmol) in H_2CCl_2 (10 mL) were added dicyclohexylcarbodiimide (0.132 g, 0.641 mmol) and a catalytic amount (4 mg) of DMAP. The resulting solution was stirred (18 h) at rt, filtered, and evaporated to a heavy oil which was chromatographed (H_2CCl_2). The oil crystallized when treated with dry hexane and gave **9** (0.075 g, 52%): mp 95−96.5 °C; IR (KBr) 1730 (C=O) cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.35 [s, 6 H, $(H_3C)_2$], 1.48 [s, 6 H, $(H_3C)_2$], 1.87 [s, 2 H, CH_2], 2.08 [s, 3 H, Ar−CH_3], 2.29 [s, 3 H, Ar−CH_3], 3.98 [s, 3 H, OCH_3], 6.61 [s, 1 H, Ar−H], 8.18 [d, 2 H, Ar−H], 8.29 [d, 2 H, Ar−H]; ^{13}C NMR ($DCCl_3$) ppm 15.17 [Ar−CH_3], 16.24 [Ar−CH_3], 27.87 [$(H_3C)_2$], 30.78 [$(H_3C)_2$], 32.13 [Ar-*C*$(CH_3)_2$], 52.51 [OCH_3], 53.33 [ArO*C*$(CH_3)_2$], 73.11 [O*C*$(CH_3)_2$], 118.09−150.80 [Ar−C], 163.97 [C=O], 166.19 [C=O]. Anal. ($C_{24}H_{28}O_5$) C, H.

Ethyl 4-[(1,1,4,4-Tetramethyl-7-methoxy-3,4-dihydro-1*H*-2-benzo[*b*]pyran-6-yl)carbonyloxy]benzoate (10). To a cloudy mixture of acid **32** (0.100 g, 0.382 mmol) and ethyl 4-hydroxybenzoate (0.095 g, 0.573 mmol) were added dicyclohexylcarbodiimide (0.118 g, 0.573 mmol) and a catalytic amount of DMAP (7 mg). The yellow solution was stirred at rt (48 h) and then filtered. Evaporation of the solution gave a heavy oil which was chromatographed (hexane:EtOAc, 3:1). An oil resulted and was treated with 0.5 mL of hexane:EtOAc (4:1) and chilled. Crystallization occurred to give solid **10** (0.110 g, 70%): mp 106.5−108 °C; IR 1722 (C=O) cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.32 [s, 6 H, $(H_3C)_2$], 1.40 [s, 3 H, *J* = 7.2 Hz, CH_3], 1.56 [s, 6 H, $(H_3C)_2$], 3.62 [s, 2 H, H_2CO], 3.94 [s, 3 H, OCH_3], 4.40 [q, 2 H, *J* = 7.2 Hz, OCH_2], 6.93 [s, 1 H, Ar−H], 7.29 [d, 2 H, Ar−H], 7.73 [s, 1 H, Ar−H], 8.12 [s, 2 H, Ar−H]; ^{13}C NMR ($DCCl_3$) ppm 14.31 [H_2C-*C*H_3], 26.79 [$(H_3C)_2$], 29.76 [$(H_3C)_2$], 34.67 [Ar*C*$(CH_3)_2$], 56.11 [Ar*C*OCH_2], 61.03 [*C*H_2−CH_3], 70.33[$ArOCH_3$], 74.86 [H_2CO], 108.79−158.29 [Ar−C], 163.59, 165.93 [C=O]. Anal. ($C_{24}H_{28}O_6$) C, H.

Ethyl 4-[(3-Methoxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalen-2-yl)carbonyloxy]benzoate (11). To a cloudy mixture of acid **35** (0.300 g, 1.14 mmol) and ethyl 4-hydroxybenzoate (0.300 g, 1.8 mmol) were added dicyclohexylcarbodiimide (0.750 g, 3.6 mmol) and a catalytic amount of DMAP (10 mg). After the resulting clear solution had been stirred at rt (24 h) and then filtered, it was evaporated to a heavy oil. Chromatography of the oil (hexane:EtOAc, 4:1) gave ester **11** (0.312 g, 67%) as a white solid: mp 104−106 °C; IR (KBr) 1722 (C=O) cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.28 [s, 6 H, $(H_3C)_2$], 1.30 [s, 6 H, $(H_3C)_2$], 1.39 [t, 3 H, *J* = 7.2 Hz, CH_2CH_3], 1.68 [2 t, 4 H, *J* = 6.0 Hz, $(CH_2)_2$], 3.89 [s, 3 H, OCH_3], 4.37 [q, 2 H, *J* = 7.2 Hz, CH_2CH_3], 6.90 [s, 1 H, Ar−H], 7.26 [d, 2 H, Ar−H], 7.93 [s, 1 H, Ar−H], 8.07 [d, 2 H, Ar−H]; ^{13}C NMR ($DCCl_3$) ppm 14.34 [CH_2CH_3], 31.65 [$(H_3C)_2$], 31.86 [$(H_3C)_2$], 33.75 [CH_2], 34.82 [CH_2], 34.84 [Ar*C*$(CH_3)_2$], 35.09 [Ar*C*$(CH_3)_2$], 56.07 [OCH_3], 61.03 [CH_2CH_3], 110.07−157.71 [Ar−C], 163.85 [C=O], 166.03 [C=O]. Anal. ($C_{22}H_{23}NO_5$) C, H.

Ethyl (2*E*,4*E*,6*E*)-7-(2,3-Dihydro-1,4-benzodioxan-6-yl)-3-methyl-2,4,6-octatrienoate (13). To a stirred suspenion of **38** (4 g, 7.53 mmol) in dry ether (40 mL) was added dropwise *n*-BuLi (14 mL, 0.906 *M*, 21.7 mmol in hexanes) over 5 min. The resulting dark red solution was stirred for 0.5 h, and then ethyl 3-methyl-4-oxocrotonate (l.3 mL, 9.15 mmol) in dry ether (10 mL) was added dropwise. After 10 h of stirring at rt, the solution was diluted (hexanes, 100 mL), filtered, and evaporated to a light yellow oil. Crystals formed upon standing and were recrystallized (absolute ethanol) to give **13** (0.683 g, 28.85%): mp 86−87 °C; IR 1690 (C=O) cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.3 [t, *J* = 7.1 Hz, 3 H, H(19)], 2.20 [s, 3 H, H(10)], 2.37 [d, *J* = 0.98 Hz, 3 H, H(15)], 4.17 [q, *J* = 7.1 Hz, 2 H, H(18)], 4.27 [s, 4 H, H(2,3)], 5.78 [bs, 1 H, H(16)], 6.33 [d, *J* = 15.1 Hz, 1 H, H(13)], 6.51 [d, *J* = 11.2 Hz, H(11)], 6.87−7.04 [m, 3 H, H(5,7,8)]. Anal. ($C_{16}H_{22}O_4$) C, H.

Methyl 4-[(2,3-Dihydro-2,2,4,4-tetramethyl-2*H*-1-benzothiopyran-6-yl)carbamoyl]benzoate (16). To a solution of amine **41** (1.6 g, 7.32 mmol) in dry benzene (90 mL) and pyridine (9.2 mL) at rt was added slowly, with stirring, freshly made methyl monoterephthaloyl chloride (2.20 g, 10.84 mmol). After stirring for 12 h (rt), the mixture was poured into water, and the resulting mixture was extracted (EtOAc, 4 × 200 mL). The combined extracts were washed with 6 N HCl (4 × 50 mL), H_2O (3 × 400 mL), saturated $NaHCO_3$ (2 × 400 mL), H_2O (50 mL), and brine (200 mL). The dried (Na_2SO_4) organic solution was evaporated to a light yellow solid which was purified via chromatography over silica gel (hexane:EtOAc:Et_2NH, 74:25:1). Recrystallization (hexane:EtOAc, 3:1) of the solid gave **16** (2.00 g, 72%): mp 154−155 °C; a further recrystallized analytical sample of **16** melted at 162−164 °C; IR (KBr) 3395−3390 (N−H), 1725 (C=O), 1690−1680 (HNC=O) cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.41 [s, 6 H, $(H_3C)_2$], 1.42 [s, 6 H, $SC(CH_3)_2$], 1.96 [s, 2 H, CH_2], 3.95 [s, 3 H, CH_3], 7.11 [d, 1 H, Ar−H], 7.33 [d, 1 H, Ar−H], 7.76 [s, 1 H, Ar−H], 7.91 [d, 2 H, Ar−H], 7.93 [bs, 1 H, Ar−H], 8.12 [d, 2 H, Ar−H], 8.13 [s, 1 H, Ar−H]; ^{13}C NMR ($DCCl_3$) ppm 31.51 [C(*C*H_3)], 32.48 [SC-(*C*$H_3)_2$], 35.94 [*C*CH_3], 42.17 [S*C*$(CH_3)_2$], 52.43 [CH_2], 54.33 [OCH_3], 118.61−143.66 [Ar−C], 164.72 [NHC=O], 166.21

[HOC═O]; MS (EI) calcd for $C_{22}H_{25}O_3SN$ [M^+] 383.1555, found 381.1552. Anal. ($C_{22}H_{25}O_3SN$) C, H, N.

***N*-(4-Methoxyoxophenyl)-4,4-dimethyl-3,4-dihydro-2*H*-benzo[*b*]pyran-6-ylhydroxamic Acid (21).** Acid **20**[7] (0.300 g, 1.40 mmol) and thionyl chloride (15 mL) were stirred at rt for 16 h. Excess thionyl chloride was evaporated under aspirator pressure, and the residual acid choride (an oil) was further dried under higher vacuum (∼2 mmHg) to remove traces of thionyl chloride. The hydroxylamine (0.253 g, 1.40 mmol) was dissolved in THF (10 mL), and then $NaHCO_3$ (0.176 g, 2.1 mmol) was added at rt. The above acid chloride was dissolved in a minimum of dry ether and then was added slowly to the solution of the hydroxylamine at 0 °C. The resulting mixture was stirred (rt) for 18 h, filtered, and evaporated in vacuo to yield a solid. Chromatography of the solid (hexanes:EtOAc, 1:10) gave **21** (0.250 g, 50%): mp 70−71 °C; IR (KBr) 3428 (O−H), 1721, 1634 (C═O) cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.13 [s, 6 H, $(CH_3)_2$], 1.78 [t, 2 H, J = 5.6 Hz, CCH_2)] 3.90 [s, 3 H, OCH_3], 4.19 [t, 2 H, J = 5.6 Hz, H_2CO], 6.68 [d, 2 H, Ar−H], 7.20 [dd, 1 H, Ar−H], 7.25 [dd, 1 H, Ar−H], 7.34 [d, 1 H, Ar−H], 7.96 [d, 2 H, Ar−H], 9.18 [s, 1 H, OH]. Anal. ($C_{20}H_{21}NO_5$) C, H. Acid **21** was used at once to obtain **7**.

2,3-Dihydro-2,6-dihydroxy-2,4,4,5,7-pentamethyl-2*H*-benzopyran (23). To a solution of 1.50 mL (4.50 mmol) of 3 M methylmagnesium bromide in ether (5 mL) was added (rt) the coumarol **22**[27] (0.100 g, 0.45 mmol) in ether (5 mL). The resulting solution was stirred for 48 h (rt). After the solution was allowed to cool to rt, a saturated solution (∼10 mL) of aqueous H_4NCl was added slowly. Separation of the layers was followed by extraction (ether, 3 × 10 mL) of the organic layer to give extracts which were washed with water and brine. After drying ($MgSO_4$), the solution was evaporated to give the lactol **23** as a colorless solid (0.57 g, 54%): mp 105.5−106.5 °C; IR (KBr) 3472, 3408 (O−H) cm^{-1} ; ^1H NMR ($DCCl_3$) δ 1.41 [s, 3 H, CCH_3], 1.58 [s, 3 H, CCH_3], 1.62 [s, 3 H, C(OH)-CH_3], 1.90 [d, 1 H, J = 14 Hz, CH_2], 1.99 [d, 1 H, J = 14 Hz, CH_2], 2.18 [s, 3 H, Ar−CH_3], 2.38 [s, 3 H, Ar−CH_3], 4.31 [s, 1 H, OH], 6.52 [s, 1 H, Ar−H]; ^{13}C NMR ($DCCl_3$) ppm 14.75 [Ar−CH_3], 15.86 [Ar−CH_3], 28.92 [$(H_3C)_2$], 29.95 [$(H_3C)_2$], 30.93 [CH_2], 32.14 [$C(CH_3)_2$], 50.07 [$H_3C(COAr)$], 95.79 [C(OH)], 117.34−147.25 [Ar−C]. MS (EI) calcd for $C_{14}H_{20}O_3$ [M^+] 236, found 236. Lactol **23** was converted to **24**.

6-Hydroxy-2,4,4,5,7-pentamethyl-4*H*-1-benzopyran (24). A mixture of lactol **23** (0.100 g, 0.42 mmol), a catalytic amount of *p*-toluenesulfonic acid (0.025 g), 4A molecular sieves (1.0 g), and toluene was boiled for 2 h. After cooling to rt and filtering, the filtrate was washed with a saturated solution of $NaHCO_3$ (∼10 mL) and then with brine. The dried (Na_2SO_4) organic solution was evaporated to yield **24** (0.082 g, 89%) as a light yellow oil: IR (neat) 3576 (O−H) cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.43 [s, 6 H, $(H_3C)_2$)], 1.85 [s, 3 H, C═C−CH_3], 2.19 [s, 3 H, Ar−CH_3], 2.33 [s, 3 H, Ar−CH_3], 4.33 [s, 1 H, OH], 4.39 [s, 1 H, C═CH_2], 6.60 [s, 1 H, Ar−H]; ^{13}C NMR ($DCCl_3$) ppm 14.38 [Ar−CH_3], 15.79 [Ar−CH_3], 18.87 [C═*C*CH_2], 31.82 [$(H_3C)_2$], 32.42 [*C*$(CH_3)_2$], 108.74 [C═*C*H], 116.12 [*C*═CCH_3], 121.91−148−30 [Ar−C]; MS (EI) calcd for $C_{14}H_{18}O_2$ [M^+] 218, found 218. This chromenol **24** was used directly to prepare heteroarotinoid **8**.

2,2,4,4,5,7-Hexamethyl-6-hydroxy-3,4-dihydro-2*H*-benzo[*b*]pyran (25). To a solution of methylmagnesium bromide (1.50 mL, 34.50 mmol, 3 M) in THF (5 mL) was added coumarol **22**[27] (0.100 g, 0.45 mmol) in THF (5 mL) at rt. The resulting solution was stirred for 4 days at reflux and was then allowed to cool. Saturated aqueous H_4NCl was added, and two layers separated. Extraction (ether, 3 × 10 mL) of the aqueous layer and combining the extracts gave an organic solution which was washed with H_2O and brine. The dried ($MgSO_4$) solution was evaporated to a colorless solid (0.086 g, 76%) of **25**: mp 62.5−64.5 °C; IR (KBr) 3458 (O−H) cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.31 [s, 6 H, $(H_3C)_2$], 1.47 [s, 6 H, $(H_3C)_2$], 1.84 [s, 2 H, CH_2], 2.17 [s, 3 H, Ar−CH_3], 2.37 [s, 3 H, Ar−CH_3], 4.25 [s, 1 H, OH], 6.50 [s, 1 H, Ar−H]; ^{13}C NMR ($DCCl_3$) ppm 14.77 [Ar−CH_3], 15.85 [Ar−CH_3], 27.84 [$(H_3C)_2$], 30.88 [$(H_3C)_2$], 31.98 [Ar$C(CH_3)_2$], 53.68 [CH_2], 72.58 [O*C*$(CH_3)_2$], 117.56−146.68 [Ar−C]; MS (EI) calcd for $C_{15}H_{22}O_2$ [M^+] 234, found 234. The sample of **25** was used at once to prepare **9**.

2-(3-Methoxyphenyl)-2-methylpropanenitrile (27). An orange mixture of KOH (20.0 g. 0.356 mol), triethylbenzylammonium bromide (TEBA; 2.0 g, 7.46 mmol), and (3-methoxyphenyl)acetonitrile (**26**; 20.0 g, 0.135 mol) was heated at 80 °C for 0.5 h. Methyl iodide (11.50 g, 0.810 mol) was added slowly over 2 h, and the resulting yellow mixture was heated (80 °C) for 1 h. Additional KOH (20.0 g, 0.356 mol) was then added along with TEBA (2.0 g, 7.46 mmol), and the new mixture was stirred vigorously for 0.5 h. Methyl iodide (11.50 g, 0.081 mol) was added over 1 h, and the resulting mixture was stirred with heating (80−100 °C) for 5 h. After cooling to rt, the reaction mixture was extracted (toluene, 5 mL). The combined organic extracts were washed with water and brine and then dried ($MgSO_4$). Evaporation of the solvent gave an oil (3.8 g, 84%) which was vacuum distilled (100−101 °C/∼2 mmHg) to a colorless oil (13.4 g, 57%) of **27**: IR (neat) 2242 C≡N cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.72 [s, 6 H, $(H_3C)_2$], 3.83 [s, 3 H, OCH_3], 6.84 [2d 1 H, Ar−H], 7.01 [t, 1 H, Ar−H], 7.05 [2d, 1 H, Ar−H], 7.31 [t, 1 H, Ar−H]; ^{13}C NMR ($DCCl_3$) ppm 29.08 [$(H_3C)_2$], 36.76 [Ar-*C*$(CH_3)_2$], 55.30 [$ArOCH_3$], 111.40−159.87 [Ar−C], 123.44 [C≡N]; MS (EI) calcd for $C_{11}H_{13}NO$ [M^+] 175, found 175. This sample of **27** was used to prepare **28**.

2-(3-Methoxyphenyl)-2-methylpropanoic Acid (28). A mixture of **27** (3.8 g, 0.0217 mol), KOH (5.00 g, 0.089 mol), diethylene glycol (30 mL), and water (5 mL) were heated at 130−140 °C for 3 days and turned brown. After cooling to rt, the solution was poured into cold water. Extraction (benzene, 8 × 40 mL) of the aqueous layer followed, and then the residual aqueous layer was made acidic (concd HCl, pH ∼ 3). The new aqueous layer was extracted (ether, 4 × 30 mL), and the combined extracts were dried ($MgSO_4$). Evaporation of the solvent gave a yellow solid which recrystallized (hexane) to a white solid **28** (3.81 g, 69%): mp 79−80 °C (lit.[28] mp 80−81.5 °C). Since spectral data were scarce on **28**, the following data were taken: IR (KBr) 3434−2538 (CO_2H), 1702 (C═O) cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.58 [s, 6 H, $(H_3C)_2$], 3.80 [s, 3 H, $ArOCH_3$], 6.80 [ddd, 1 H, Ar−H], 6.95 [t, 1 H, Ar−H], 6.99 [ddd, 1 H, Ar−H], 7.25 [t, 1 H, Ar−H]; MS (EI) calcd for $C_{11}H_{14}O_3$ [M^+] 194, found 194. Acid **28** was converted directly to **29**.

2-(3-Methoxyphenyl)-2-methyl-1-propanol (29). To a mixture of $LiAlH_4$ (2.23 g, 58.9 mmol) in dry THF (19 mL) was added dropwise acid **28** (3.81 g, 19.6 mmol) in dry THF (5 mL) over 0.5 h. After being heated at reflux (48 h), the resulting mixture was allowed to cool to rt and was then treated *cautiously* with ethanol/water to destroy residual $LiAlH_4$. A white solid formed and was filtered, and the remaining aqueous layer was extracted (ether, 5 × 30 mL). The combined extracts were washed with brine, dried ($MgSO_4$), and evaporated to a colorless oil **29** (2.6 g, 75%): IR (neat) 3392 (O−H) cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.32 [s, 6 H, $(H_3C)_2$], 3.59 [s, 2 H, H_2COH], 3.81 [s, 3 H, $ArOCH_3$], 6.75 [2 d, 1 H, Ar−H], 6.93 [t, 1 H, Ar−H], 6.97 [2 d, 1 H, Ar−H], 7.27 [t, 1 H, Ar−H]; MS (EI) calcd for $C_{11}H_{16}O_2$ [M^+] 180, found 180. The sample of **29** was used immediately to prepare ether **30**.

1,1,4,4-Tetramethyl-6-methoxy-3,4-dihydro-1*H*-2-benzopyran (30). A solution of alcohol **29** (0.989 g, 5.6 mmol), acetone (30 mL), and concd HCl (10 mL) was heated and stirred at 45−50 °C for 2 h and at rt for 12 h. The deep yellow solution was allowed to cool to rt and was then poured into ice−water. Two layers were separated, and the organic layer was extracted (ether, 5 × 20 mL). The combined extracts were washed with water (30 mL), saturated $NaHCO_3$ (30 mL), and brine and then was dried ($MgSO_4$). Evaporation of the solvent gave a colorless oil **30** (0.92 g, 75%) which was used directly to prepare **31**. Spectral data for **30** were as follows: ^1H NMR ($DCCl_3$) δ 1.26 [s, 6 H, $(H_3C)_2$], 1.51 [s, 6 H, $(H_3C)_2$], 3.58 [s, 2 H, H_2CO], 3.80 [s, 3 H, $ArOCH_3$], 6.73 [dd, 1 H, Ar−H], 6.82 [d, 1 H, Ar−H], 6.99 [d, 1 H, Ar−H]; MS (EI) calcd for $C_{14}H_{20}O_2$ [M^+] 220, found 220.

7-Aceto-1,1,4,4-tetramethyl-6-methoxy-3,4-dihydro-1*H*-2-benzopyran (31). To a solution of **30** (0.989 g, 4.18 mmol), acetyl chloride (0.74 mL, 10.0 mmol), and H_3CNO_2 (15 mL)

was added very slowly and portionwise $AlCl_3$ (1.33 g, 10.0 mmol) over 1 h. The resulting brown mixture was stirred at rt for 24 h and was then poured cautiously into ice−water (50 mL). Dilute HCl (2 M, ∼5 mL) was added *cautiously* to destroy residual $AlCl_3$. The layers were separated, the aqueous layer was extracted (H_2CCl_2, 3 × 10 mL), and the combined organic extracts—original layer were washed with H_2O (20 mL), saturated aqueous $NaHCO_3$ (25 mL), and brine. Evaporation of the solvent gave a solid which was by purified by chromatography (H_2CCl_2) and yielded a colorless solid **31** (0.562 g, 51%): mp 115−117 °C; IR (KBr) 1662 (C═O) cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.29 [s, 6 H, $(H_3C)_2$], 1.52 [s, 6 H, $(H_3C)_2$], 2.60 [s, 3 H, $C(O)CH_3$], 3.59 [s, 2 H, OCH_2], 3.91 [s, 3 H, $ArOCH_3$], 6.85 [s, 1 H, Ar−H], 7.50 [s, 1 H, Ar−H]; MS (EI) calcd for $C_{16}H_{22}O_3$ [M^+] 262, found 262. Ketone **31** was used at once to prepare acid **32**.

1,1,4,4-Tetramethyl-6-methoxy-3,4-dihydro-1*H*-2-benzopyran-7-carboxylic Acid (32). To ketone **31** (0.560 g, 0.216 mmol) was added a solution of LiOCl (Bioguard-LiOCl 29%, inert 71%)/Clorox [5 g LiOCl (Bioguard), 20 mL Clorox] and 95% ethanol (10 mL) in the normal manner.[40] The resulting light yellow solution was boiled (24 h) and then allowed to cool to rt. The basic solution was extracted (ether, 3 × 15 mL), and the combined extracts were washed with water (10 mL) and brine and then were dried ($MgSO_4$). The original aqueous layer was acidified (2 M HCl, 10 mL, ∼pH 3) and then extracted (ether, 4 × 20 mL). The combined organic layers were washed with water (20 mL) and brine and then dried ($MgSO_4$). Evaporation of the solvent gave a colorless oil which crystallized (EtOAc) to give **32** (0.420 g, 21%): mp 147−149 °C; IR 3443−2538 [C(O)O−H], 1691 [C═O] cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.30 [s, 6 H, $(H_3C)_2$], 1.54 [s, 6 H, $(H_3C)_2$], 3.61 [s, 2 H, H_2CO], 4.07 [s, 3 H, $ArOCH_3$], 6.93 [s, 1 H, Ar−H], 7.89 [s, 1 H, Ar−H]; MS (EI) calcd for $C_{15}H_{20}O_4$ [M^+] 264, found 264. The acid **32** was used directly to obtain the target compound **10**.

1-(3-Hydroxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalen-2-yl)ethanone (34). To a solution of ether **33**[32] (0.050 g, 0.23 mmol), acetyl chloride (0.049 g, 0.69 mmol), and H_3CNO_2 (5 mL) was added slowly and portionwise $AlCl_3$ (0.092 g, 28.9 mmol) over 1 h. After stirring at rt (4 h), the brown solution was poured into ice−water (10 mL). Two layers were separated, and the aqueous layer was extracted (ether, 2 × 10 mL). The combined organic layers were washed with H_2O (20 mL), saturated aqueous $NaHCO_3$ (15 mL), and brine. After drying ($MgSO_4$) and evaporation of the solvent from the solution, ketone **34** (0.045 g, 75%) was obtained as a light yellow solid: mp 96−98 °C; IR (KBr) 1664 cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.27 [s, 6 H, $(H_3C)_2$], 1.30 [s, 6 H, $(H_3C)_2$], 1.68 [2t, 4 H, J = 6.0 Hz, $(H_2C)_2$], 2.59 [s, 3 H, CH_3], 3.89 [s, 3 H, OCH_3], 6.85 [s, 1 H, Ar−H], 7.73 [s, 1 H, Ar−H], 11.87 [s, 1 H, OH]; MS (EI) calcd for $C_{17}H_{24}O_2$ [M^+] 260, found 260. Ketone **34** was converted to acid **35**.

3-Methoxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-carboxylic Acid (35). A mixture of ketone **34** (0.900 g, 3.46 mmol), a solution of LiOCl and Clorox [40 g LiOCl (Bioguard), 80 mL Clorox], and 95% ethanol (45 mL) was boiled for 24 h and then allowed to cool to rt. An aqueous solution of $Na_2S_2O_5$ (25%, 35 mL) was slowly added to the reaction mixture which was then acidified with 6 M HCl (10 mL, pH ∼ 3). Extraction (ether, 4 × 20 mL) of this mixture and then washing of the combined extracts with H_2O (20 mL) and brine gave a new solution which was dried ($MgSO_4$). Evaporation of the solvent gave a very light yellow solid **35** (0.420 g, 44%): mp 136.5−138 °C; IR (KBr) 3269 (broad) [C(O)O−H], 1731 [C═O] cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.28 [s, 6 H, $(H_3C)_2$], 1.31 [s, 6 H, $(H_3C)_2$], 1.69 [t, 4 H, J = 5 Hz, CH_2], 4.06 [s, 3 H, OCH_3], 6.93 [s, 1 H, Ar−H], 8.12 [s, 1 H, Ar−H]; ^{13}C NMR ($DCCl_3$) ppm 31.58 [$(H_3C)_2$], 31.67 [$(H_3C)_2$], 33.83 [CH_2], 34.60 [CH_2], 34.65 [Ar$C(CH_3)_2$], 35.09 [Ar$C(CH_3)_2$], 56.47 [OCH_3], 109.15−155.53 [Ar−C], 165.60 [C═O]; MS (EI) calcd for $C_{16}H_{22}O_3$ [M^+] 262, found 262. Acid **35** was used directly to prepare ester **11**.

2-(2,3-Dihydro-1,4-benzodioxan-6-yl)-3-buten-2-ol (37). To a solution of commercial ketone **36** (4.0 g., 22.45 mmol) in THF (25 mL) was added dropwise vinylmagnesium bromide [prepared from Mg (1.52 g, 62.53 mmol) and vinyl bromide (10 g, 93.45 mmol) in THF (10 mL)] over 0.25 h. After stirring at rt for 10 h, the mixture was cooled in a water bath, and a saturated aqueous solution (20 mL) of H_4NCl was added dropwise to effect decomposition. Separation and extraction (ether, 4 × 50 mL) of the aqueous layer gave a solution of combined extracts which was washed with saturated, aqueous NaCl. Drying of the organic solution and evaporation of the solvent gave slightly crude **37** (4.78 g, 103%) as a yellow oil: IR (neat) 3700−3100 (O−H) cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.60 [s, 3 H, H(10)], 4.23 [s, 4 H, H(2,3)], 5.11 [dd, J_{cis} = 10.6 Hz, J_{gem} = 1.08 Hz, 1 H, H(12)], 5.27 [dd, J_{trans} = 17.2 Hz, J_{gem} = 1.08 Hz, J_{gem} = 1.08 Hz, 1 H, H(12)], 6.12 [dd, J_{trans} =17.3 Hz, J_{cis} = 10.6 Hz, H (11)] 6.8−6.99 [m, Ar−H, 3 H, H(5,7,8)]; HRMS calcd for $C_{12}H_{14}O_3$ [M^+] 206.0942, found 206.0938. This sample of **37** was used without further purification to prepare salt **38**.

[3-(2,3-Dihydro-1,4-benzdioxan-6-yl)-2-buten-1-yl]triphenylphosphonium Bromide (38). To a stirred suspension of $Ph_3P{\cdot}HBr$ (2.61 g, 4.92 mmol) in dry methanol (20 mL) was added dropwise alcohol **37** (1.01 g, 4.92 mmol) in dry methanol (30 mL) over 10 min at rt under N_2. After stirring at rt for 10 h, the suspension was concentrated to about 5 mL and was then transferred to a large beaker. Dry ether was added slowly with stirring to complete the precipitation. Filtration gave a solid which was washed (dry ether) and then was dissolved in dry methanol (∼15 mL). Ether was added to cloudiness, and the resulting mixture was chilled in the freezer. Filtration and drying the solid gave fine, white crystals of **38** (2.22 g, 84.92%): mp 234−235 °C; ^1H NMR ($DCCl_3$) δ 1.58 [d, 3 H, H(01)], 4.22 [s, 4 H, H(2,3)], 4.84 [dd, J_{HP} = 15.1 Hz, J_{HH} = 8.1 Hz, H(12)], 5.57 [m, 1 H, H(11)], 6.88 [m, 3 H, H(5,7,8)], 7.65−7.93 [m, 14 H, Ar−H, $P(C_6H_5)_3$]. In view of the rarity of such salts, an elemental analysis was obtained and resulted in the identification of a monohydrate. Anal. ($C_{30}H_{28}O_2PBr{\cdot}H_2O$) C, H. Salt **38** was used directly to obtain **13**.

2,2,4,4-Tetramethyl-6-nitrothiochroman (40). To a solution of thioether **39**[5] (5.13 g, 24.8 mmol) in freshly distilled Ac_2O (5 mL) at 0 °C was added slowly a mixture of cold, concd HNO_3 (3.54 mL) and Ac_2O (9 mL) over 10 min. After being stirred for 2 h, the reaction mixture was poured into a solution of saturated, aqueous $NaHCO_3$. Extraction (H_2CCl_2, 3 × 40 mL) of the water layer and combining the extracts and organic layer gave a solution which was washed with water and brine and then was dried (Na_2SO_4). Evaporation of the solvent gave a solid which was recrystallized (hexane) to yield **40** (1.6 g, 27%): mp 103−107 °C; ^1H NMR ($DCCl_3$) δ 1.10 [s, 3 H, $(H_3C)_2$], 1.37 [s, 3 H, $(H_3C)_2$], l.52 [s, $SC(CH_3)_2$], 1.56 [s, 3 H, $SC(CH_3)_2$], 2.03 [m, 3 H, CH_2], 8.01 [d, 1 H, Ar−H], 8.24 [d, 2 H, Ar−H]. The sample of **40** was used directly to prepare amine **41**.

2,2,4,4-Tetramethyl-6-aminothiochroman (41). To chroman **40** (0.800 g, 3.18 mmol) in acetic acid (29 mL) and water (6 mL) was added dropwise with stirring $TiCl_3$/HCl (33.00 g, 21.39 mmol). After stirring for 2 h (rt), the reaction mixtrure was cooled (0 °C), and NaOH (30%, 130 mL) was added slowly. Extractions (EtOAc, 4 × 35 mL, and H_2CCl_2, 2 × 40 mL) of the mixture followed, and combining the extracts and organic layer gave a solution which was washed with H_2O and saturated $NaHCO_3$ (2 × 50 mL). The dried (Na_2SO_4) solution was evaporated to an oil which was separated via chromatography over silica gel (hexane:H_2CCl_2, 1:1) and led to **41** as a white solid (0.352 g, 50%): mp 57−59 °C; IR (KBr) 3450, 3360 (N−H) cm^{-1}; ^1H NMR ($DCCl_3$) δ 1.36 [s, 6 H, $(H_3C)_2$], 1.39 [s, 6 H, $SC(CH_3)_2$], 1.90 [s, 2 H, CH_2], 3.50 [bs, 2 H, NH_2], 6.44 [d, 1 H, Ar−H], 6.75 [s, 1 H, Ar−H], 9.92 [d, 1 H, Ar−H]. The sample of **41** was used directly to prepare **16**.

In Vitro Growth Inhibition. Cultures were plated with 96-well microtiter plates in volumes of 150 μL at a concentration of 2500 cells/well. The next day, retinoids were added at 4X concentrations in 50 μL of media resulting in 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M final concentrations of each retinoid. Control cultures were treated with the same volume of DMSO. After 3 days of treatment, the cell density in each well was determined by fixing the cells in trichloroacetic acid and

staining the cytoplastic proteins with sulforhodamine B (SRB). After rinsing, the SRB was solubilized in TrisHCl, and the optical density of each culture was determined with a MR600 microtiterplate reader. Each experiment was performed in triplicate, and the three values for each treatment were averaged. The average OD of the treated cultures was divided by that of the control cultures treated with solvent alone. To determine the percent growth inhibition, this ratio was subtracted from 1 and multiplied by 100.

In Vivo Tumor Xenographs. Cells in log phase growth were harvested by trypsinization, resuspended in MEM culture medium, and centrifuged at 3000 rpm for 10 min. The pellets were resuspended in MEM culture medium at a concentration of 1×10^7 cells/mL before implantation into mice. Five-week old female athymic *nu/nu* mice (Harlan Sprague-Dawley, Indianapolis, IN) were housed in a laminar flow room under sterile conditions at 83−85 °F. The mice were quarantined for 1 week prior to the beginning of the study and were allowed access to autoclaved food (Purina 5001 mouse/rat sterilizable diet, St. Louis, MO) and water ad libitum. Animals were injected with 1×10^7 cells into the right scapular region with a 24-gauge needle/1 cm^3 tuberculin syringe (Becton Dickinson, Rutherford, NJ). Twenty-four hours after tumor implantation, animals were randomized into 4 treatment groups of 5 animals each. Retinoids were administered daily po beginning 5 days after tumor implantation with a 20-gauge intragastric feeding tube (Popper & Sons, New Hyde Park, NY), 5 days/week, at doses of 10 or 20 mg/kg/day in 0.1 mL of super-refined sesame oil (Corda, Inc., Parisppany, NJ). Tumors were measured with calipers thrice weekly, and tumor volumes were calculated using the formula: volume = length × width × heigth. Animal weights were recorded thrice weekly, and clinical signs of overall health status and cutaneous toxicities were recorded weekly.

Receptor Expression. RNA was isolated using the Quick-Prep Kit (Amershsam Pharmacia Biotech, Piscataway, NJ), and cDNA was synthesized with Superscript reverse transcriptase (GibcoBRL, Gaithersburg, MD) according to the manufacturer's instructions. The specific primers and annealing conditions used for the PCR reactions were previously described and demonstrated to be specific for the individual receptor subtypes.[41] The RXR expression was not evaluated because a human RXRγ gene has not been cloned. All PCR reactions were performed under the same conditions in a 50-μL reaction volume containing 5 μL of cDNA from reverse transcription, 1.25 U of Taq DNA polymerase, and the buffer provided. Initial denaturation was performed at 94 °C for 5 min, followed by 35 cycles of 45 s at 94 °C, 45 s at the indicated annealing temperature, and 1 min at 72 °C with an ending of 1 5-min cycle at 72 °C. Negative control reactions consisted of rt-PCR reactions without added RNA.

Receptor Activation. The CV-1 cells were plated in 6-well culture plates at a density of 7.5×10^4 cells/well and, after 24 h, were cotransfected with a retinoic acid receptor expression vector (pECE-hRARα, pECE-hRARβ, pECE-hRARγ, pSG5-mRXRα, pSG5-mRXRβ, or pSG5-mRXRγ), a retinoic acid responsive reporter plasmid (βRARE-tk-CAT), and a transfection control plasmid (pJATLac) using FuGENE6 transfection reagent (Boehringer Mannheim Corp., Indianapolis, IN). The βRARE-tkCAT plasmid contained the chloramphenicol acetyltransferase gene (CAT) driven by the thymidine kinase (tk) promoter and the RARβ retinoic acid response element (RARE). The pJATLac plasmid contained the β-galactosidase (βGal) gene driven by the βactin promoter. About 16−18 h after transfection, retinoids or DMSO (solvent) was added to the cells. The retinoids were administered at 10-fold dilutions of concentrations ranging from 10^{-5} to 10^{-10} M. The concentration of DMSO in all treated and control cultures was 0.01% which was not cytotoxic to the cells. After incubation for 24 h, cell lysates were prepared and assayed for reporter expression using CAT and βGal ELISA kits (Boehringer Mannheim Corp., Indianapolis, IN) according to the manufacturer's instructions. The CAT expression levels were corrected for transfection efficiency by dividing by the βGal expression levels. Each experiment was performed in triplicate and repeated at least twice.

Anti-AP-1 Activity. The SCC-2 cells were plated at 3×10^5 cells/well in 6-well plates 24 h before transfection. The cultures were cotransfected with a CAT reporter plasmid driven by the collagenase promoter (Col-CAT) and the pJAT-Lac plasmid DNA using FuGENE6 transfection reagent (Boehringer Mannheim Corp., Indianapolis, IN) according to the manufacturer's instructions. After incubation for 24 h, the media was replenished, and retinoids were added at a concentration of 10^{-5} M. After another 48 h, CAT and βGal expressions were quantitated as described above. All values were divided by the value of control cultures treated with DMSO to determine the percent activities. Each experiment was performed in duplicate and repeated twice.

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