Azulenic Retinoids: Novel Nonbenzenoid Aromatic Retinoids with Anticancer Activity

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Several novel azulene-containing retinoids were prepared and evaluated for their ability to suppress carcinogen-induced neoplastic transformation and to concomitantly up-regulate gap junctional communication in the *in vitro* mouse fibroblast C3H/10T1/2 cell bioassay. The azulenic retinoids were divided into two groups: compounds **1–6** were modeled after retinoic acid with flexible polyenic side chain whereas retinoids **7–13** featured a benzoic acid moiety analogous to the prototypic retinobenzoate (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB). Within this latter group the side chains for compounds **7**, **10**, and **11** were attached at the 1-, 2-, and 8-positions of the azulenic terminus, respectively. Biological activities were determined for all the new compounds. Two of these novel retinoids, azulenic retinobenzoic acid derivatives **7** and **11**, were completely effective inhibitors of transformation at 10^{-6} M. The most active azulenic retinoids also enhanced gap junctional communication in untransformed cells; this was associated with up-regulated expression of connexin 43, a structural protein of the gap junction. Two fluorinated analogs were also tested. The azulenic fluoro acid **5** was found to be more potent than the trifluoromethyl analog **6**. Azulenic analogs with hydroxyl or carboxaldehyde side chain functional groups were ineffective transformation inhibitors. In general, azulenic retinobenzoic acid analogs structurally akin to TTNPB were more effective than flexible side chain analogs related to retinoic acid.

Introduction

Retinoids, the natural and synthetic derivatives of vitamin A,[1] are potent modulators of growth and differentiation. The parent molecule, retinol, is metabolized in target tissues to the physiologically active compounds *all-trans*- and 9-*cis*-retinoic acid. These exert their effects by binding to nuclear retinoic acid receptors (RARs) or other retinoid receptors (RXRs), which alter the transcriptional activity of retinoid-responsive genes.[2] A family of receptors exist which differ in their tissue distribution, ligand specifity, and ability to regulate target genes.[2] Synthetic retinoids have been described that can preferentially activate individual receptors.[3,4] It may be expected that new members of this family are yet to be discovered with affinity for novel retinoids.[5]

Retinoids are used extensively in dermatology for a variety of conditions.[6] They also show activity as cancer preventive agents (as cancer chemopreventives), both experimentally and clinically, when applied prior to frank malignancy.[7] Recently *all-trans*-retinoic acid has revolutionized the treatment of promyelocytic leukemia (as a cancer chemotherapeutic agent) by causing terminal differentiation of the malignant cells.[8]

The limiting factor for the clinical use of retinoids is toxicity, which for the most part represents an overexpression of the physiological functions of the retinoids. The ability to target retinoids to specific genes in specific tissues would be expected to circumvent the widespread toxicity observed after systemic administration. This may be a realistic goal in view of the diversity in expression and ligand-binding characteristics of the nuclear retinoid receptors discussed above. Here we describe the synthesis of a new class of nonbenzenoid aromatic analog of retinoic acid, the azulenic retinoids (Figure 1), some of which possess biological activity as cancer chemopreventive agents in a widely used model system of carcinogenesis.

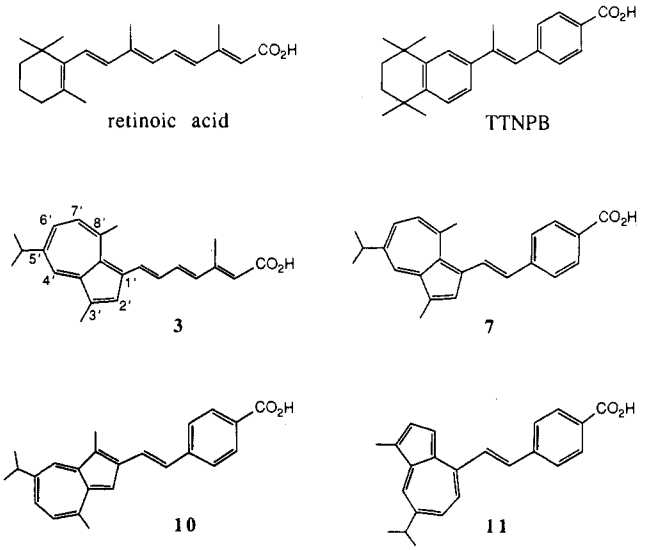

Figure 1. Retinoic acid, (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB), type I azulenic retinoid **3** containing a flexible side chain and type II retinobenzoic acid analogs **7**, **10**, and **11**.

Results and Discussion

Chemistry. The chemical, electronic, and biological properties of azulene, a strikingly beautiful blue nonalternant aromatic hydrocarbon, are uniquely different

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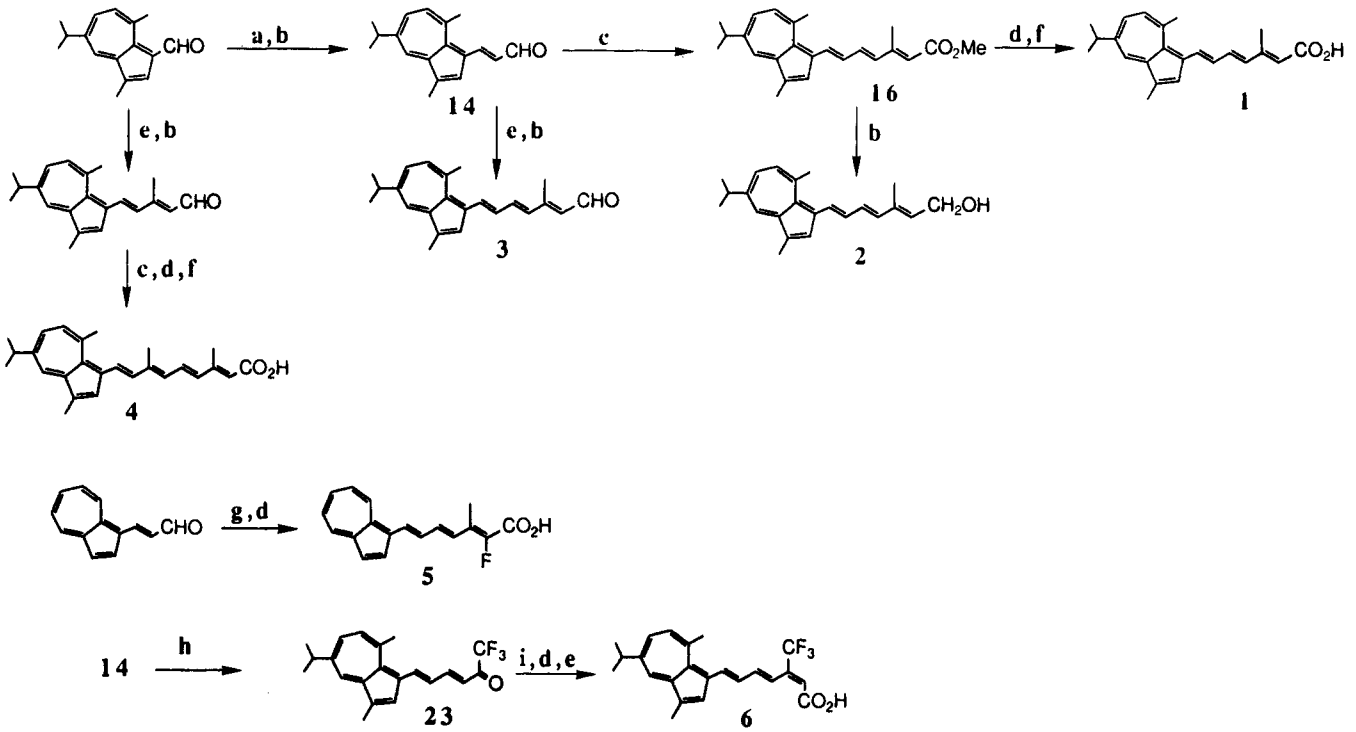
Scheme I[a]



[a] (a) $(EtO)_2P(O)CH_2CN$, LDA, THF; (b) $(^iBu)_2AlH$; (c) $(EtO)_2P(O)CH_2C(CH_3)=CHCO_2Me$, **15**, LDA, THF; (d) KOH, MeOH; (e) $(EtO)_2P(O)CH_2C(CH_3)=CHCN$, **17**, LDA, THF; (f) H_3O^+, Cl^-; (g) $(EtO)_2P(O)CH_2C(CH_3)=CFCO_2Et$, **21**, LDA, THF; (h) CF_3COCH_3, piperidine, acetic acid; (i) $(EtO)_2P(O)CH_2CO_2Et$, LDA, THF.

from those of the alternant aromatic hydrocarbon naphthalene, to which it is both isomeric and isoelectronic.[9] Because of its unique electronic distribution, azulene undergoes facile electrophilic substitution at the 1-position of the five-membered ring, often without the intervention of Lewis acid catalysis.[10,11] On the other hand, nucleophilic substitution takes place on the seven-membered ring at the relatively electropositive 4- and 6-positions.[10,12]

From antiquity azulene-containing plants such as calamus, chamomile, galbanum and yarrow have been prized for their medicinal value. Some of the more useful pharmacological properties include antibacterial, antiinflammatory, and wound-healing activities.[13] Recently, guaiazulene derivatives isolated from the marine gorgonian *Acalycigorgia* were found to possess antitumor, immunomodulatory, and antifungal activity.[14] In addition to medicinal applications, azulenic compounds are also potentially useful for technological applications related to their long-wavelength (near-infrared) light-absorption properties.[15]

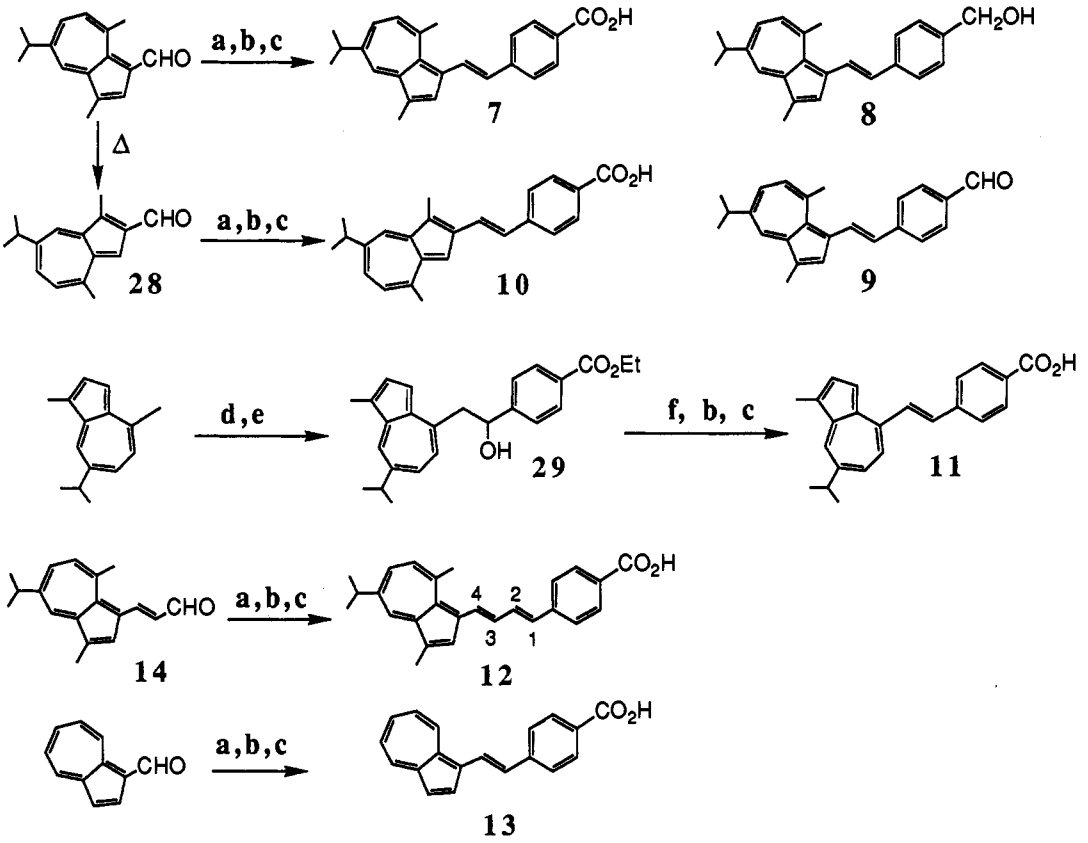
We recently described the use of novel azulene-containing retinal analogs for the regeneration of extraordinarily red-shifted analogs of bacteriorhodopsin.[16] Attenuation of their absorption maxima deep into the near-infrared (830 nm) was ascribed to the intrinsic large red shift of the protonated Schiff base form of the chromophore within the binding site of the protein. Additional bathochromic shift was attributed to charge stabilization by delocalization into the azulene portion of the chromophore to form an azulenylium carbocation intermediate. Such specific resonance stabilization is unique to nonalternant hydrocarbons such as azulene when appropriately substituted (1- vs the 2-position). Thus, bacteriorhodopsin analogs regenerated from 2-substituted azulenic retinals do not exhibit especially red-shifted absorption maxima as do their 1-substituted counterparts.[17] Azulenic retinals have also been successfully incorporated into a retinal-deficient strain (FN68) of the light-sensitive unicellular eukaryotic alga *Chlamydomonas reinhardtii* with restoration of phototactic response to light of near-infrared wavelengths.[18]

Encouraged by these results, which demonstrated the biological utility of azulenic retinal analogs, we undertook the preparation of a representative set of new azulene-containing retinoid analogs **1–13** for evaluation as potential cancer chemopreventive agents. Standard synthetic methodologies previously employed for the preparation of vitamin A derivatives were used throughout this study (Schemes I and II).[19]

The azulenic retinoids were divided into two main groups. The first set of compounds, **1–6** (type I), depicted in Scheme I, contained an azulenic moiety as the nonpolar terminus of the molecule appended to a flexible polyenic side chain. In addition to the carboxylic acid polar terminus, analogs of retinol and retinal, **2** and **3**, respectively, were also included in this survey. The second set of arotinoids, retinobenzoic acid analogs **7–13** (type II), featured an azulenic nonpolar end group separated from the carboxyl polar terminus by a double bond and benzene ring (Scheme II). Benzyl alcohol derivative **8** and benzaldehyde **9** were also evaluated for potential inhibition activity.

For both type I and II arotinoids, the side chain was varied in order to determine longitudinal length requirements (**1** vs **4** and **7** vs **12**). Furthermore, hydrophobic end group requirements were evaluated by comparing the unencumbered azulenic retinoid **13** to the more highly alkylated guaiazulenic derivative **7**. Fluorinated azulenic arotinoids **5** and **6** were also included in this survey since fluorine is known to markedly influence the anticancer and vision-related activities of retinoids.[20] The final structural modification represented by retinoids **7**, **10**, and

Scheme II[a]



[a] (a) 4-$(EtO)_2P(O)CH_2C_6H_4CO_2Me$, **24**, LDA; (b) KOH, MeOH; (c) H_3O^+, Cl^-; (d) LDA; (e) 4-$OHCC_6H_4CO_2Et$; (f) $POCl_3$, pyridine, benzene.

11 involved variation of the site of attachment of the ethenylbenzoic acid side chain to the azulene nucleus (Figure 1). These changes afforded retinoids with substantially different molecular shapes and electronic properties. We were interested in what, if any, effect these changes of molecular dimensions or other potential electrostatic interactions would have on their respective anticancer activities.

Synthesis. The retinoids depicted in Scheme I were routinely assembled by phosphonate olefinations using C2- and C5-reagents such as the ester **15** and nitrile **17**. The starting materials were azulene-1-carboxaldehyde and guaiazulene-1-carboxaldehyde, both readily accessible from their respective commercially available parent hydrocarbons by formylation with triethyl orthoformate in the presence of boron trifluoride etherate.[21] The Horner reaction of guaiazulene-1-carboxaldehyde with C2-phosphononitrile gave the homologous propenenitrile, a green solid, in modest yield (56%). Partial reduction with diisobutylaluminum hydride (DIBAL) afforded propenal **14**, previously prepared by a different method,[22] in 60% yield. Thereafter, treatment of aldehyde **14** with the lithio derivative of C_5-phosphonate ester **15** afforded the desired trienoic ester **16** as purple platelets (84%). Lastly, base hydrolysis of the ester by warming with aqueous methanolic KOH afforded trienoic acid **1** as a brown solid. Vitamin A analog **2**, a green solid, was obtained from ester **16** by straightforward DIBAL reduction.

Chain-extension of enal **14** with the lithio derivative of C_5-phosphononitrile **17** gave the trienenitrile, which upon reduction with DIBAL gave retinal analog **3**.[16] Tetraenoic acid **4** was assembled by sequential chain extension of guaiazulene-1-carboxaldehyde using phosphononitrile **17** followed by phosphono ester **15**.

Azulenic fluoro acid **5** was prepared by C5-extension using the fluorinated phosphonate **21**[20e] and 3-(1-azulenyl)-propenal,[22] prepared from the corresponding nitrile by DIBAL reduction in good yield (84%). The preparation of trifluoromethylated guaiazulenic trienoic acid **6** was carried out by the piperidinium acetate-catalyzed aldol condensation[23] of guaiazulene-1-carboxaldehyde with trifluoroacetone to give trifluoromethyl dienone **23** followed by normal carbethoxyolefination and subsequent hydrolysis.

The type II azulenic retinoids, featuring a double bond and benzoic acid group in place of the normal flexible polyolefinic side chain, were prepared as depicted in Scheme II. Benzoic acid analog **7** was obtained by the condensation of guaiazulene-1-carboxaldehyde with the lithio derivative of the aromatic phosphonate methyl 4-[(diethylphosphono)methyl]benzoate, **19**,[24] followed by conventional saponification. Benzyl alcohol **8** was obtained by DIBAL reduction of the same ester and benzaldehyde derivative **9** was prepared in a manner similar to the preparation of retinal analog **3** from its benzonitrile precursor.

The starting material for benzoic acid analog **10**, guaiazulene-2-carboxaldehyde, was prepared in low isolated yield (8%) by thermal isomerization of the corresponding isomeric 1-carboxaldehyde.[25] We found this simple isomerization protocol to be expedient in comparison to other synthetic procedures.[26] Thereafter, the synthesis was completed by olefination with phosphonate **19** followed by base hydrolysis to give acid **10** as a dark green solid.

Acid **11**, featuring the ethenylbenzoic acid moiety appended to the 8-position of azulene, was prepared by the reaction of the lithio derivative of guaiazulene

("lithioguaiazulenide")[27] with ethyl 4-formylbenzoate to give hydroxy ester **29** in high yield. Dehydration of **29** using $POCl_3$ and pyridine in benzene followed by ester hydrolysis completed the synthesis. This reaction was complicated by the formation of a considerable amount of nonpolar byproduct, which was tentatively identified as the chloro substitution product (data not shown).

Benzoic acid **12** contained an extra double bond in comparison to acid **7**. The starting material for **12** was once again propenal **14**, which was chain-extended using phosphonate **24**. And lastly, the unsubstituted azulenic acid **13** was prepared from azulene-1-carboxaldehyde and **24** in a manner similar to the preparation of **7**.

With the exceptions of retinoids **3** and **5**, which were tested as cis/trans-isomeric mixtures about the terminal double bond, all other compounds were purified to configurational homogeneity by multiple recrystallizations or column chromatography on silica gel. All chemical structures were spectroscopically confirmed.

Biological Activity. Compounds were tested in two groups because of logistical problems in the handling of larger number of cell cultures. Separate solvent-only, and carcinogen-treated control cultures as well as TTNPB-positive controls were included with each experimental group and are listed separately. Several azulenic compounds were active as inhibitors of carcinogen-induced neoplastically transformed foci. In addition, as previously observed with other retinoids, active compounds caused up-regulation of gap junctional communication. As shown in Table I, in which the biological properties of all tested compounds are summarized, these two actions were tightly associated. It will be noted that none of the tested compounds was as potent an inhibitor as was TTNPB of carcinogen-induced (3-methylcholanthrene) neoplastic transformation in the 10T1/2 system.[28] Nor did any of the new retinoids enhance gap junctional communication (gjc) as well as TTNPB, one of the most potent retinoids yet synthesized.[32]

Of the novel retinoids tested, the rank order of activity, based on all three assays, is compound **11** > **5** > **7** > **10** = **13**. Compound **11** completely inhibited transformation at 10^{-6} M, at which concentration communication, as measured by dye-transfer, was stimulated almost 6-fold over controls.

A major increase in connexin 43, the only junctional protein known to be expressed in 10T1/2 cells,[29] was also detected by immunoblotting (Western blots) of protein extracted from cells treated with retinoids active as inhibitors of transformation and as stimulators of gap junctional communication (Figure 2). Thus, as for the other retinoids previously tested in this system, increased communication is driven by increased gene expression of connexin 43.[29] We have proposed that this increased communication places carcinogen-initiated cells in a communication network governed by nonproliferating normal cells that acts to suppress their neoplastic transformation.[28]

For compounds **10** and **13** interpretation of potency is made difficult by a flat dose/response curve in the transformation assays. This may simply be due to sampling error associated with the lower frequency of transformants produced by MCA treatment in the second transformation assay (0.66 foci/dish, for compounds **4–6**, **10–13**) than the first assay (1.05 foci/dish, compounds **1–3**, **7–9**).

For vitamin A analog **2** and the aldehydic derivative **9**, doses of 10^{-8} M resulted in more transformants than in solvent-treated controls. This phenomenon has been previously reported for retinoic acid, where comparable low doses were found to lead to inhibition of gap junctional communication, while high doses inhibited transformation and stimulated communication.[28] We did not assay for communication in cultures treated with low doses of azulenic retinoids, but predict that a similar biphasic dose/response would be observed. As shown in Table I, inhibition of transformation does not appear to be associated with cytotoxicity since the retinoids were not toxic to normal 10T1/2 cells and they were administered after removal of the carcinogen, long after the process of initiation has occurred.[30] The conclusion is strengthened by our previous studies which demonstrated that the inhibition of transformation by retinoids was reversible upon drug withdrawal.[30]

Factors Affecting Retinoid Activity. From several comprehensive structure–activity relationship studies involving numerous retinoid analogs have emerged important structural requirements for retinoid anticancer activity.[31] These structural guidelines specify the critical molecular dimensions, hydrophobicity, and end group polarity to achieve maximal inhibition. The greatest antiproliferative potency has been realized for certain structurally constrained analogs typified by TTNPB.[32] Other aromatic retinoids featuring an azo linkage[33] in place of the propenyl side chain, heteroatom-containing arotinoids[34a] as well as benzoic acid analogs featuring amide linkages[34b] or trimethylsilyl and trimethylgermyl substituents[34c] are apparently less active than TTNPB.

To compare the effect of the oxidation state of the side chain polar end group, azulenic analogs of retinol and retinal (**2**, **3**, **8**, and **9**) were also included in this study. These compounds were devoid of inhibitory activity, although cell plating efficiency increased for as yet unknown reasons. These results are entirely consistent with known structural trends for retinoidal anticancer activity.[31]

For all the other azulenic retinoids with carboxylic acid polar head groups, those with retinoic acid-like flexible side chains (type I) were less potent than the benzoic acid derivatives (type II). Incorporation of the aromatic ring into the side chain serves to truncate the molecule (equivalent to the introduction of a 12-s-cis linkage in RA) as well as to eliminate comformational flexibility near the polar terminus. This structural constraint has also been documented for other analogs, including TTNPB.[31]

The effect of alkyl substituents on the azulene could be evaluated for retinoids **7**, containing a 1-guaiazulenyl end group, vs **13**, an unsubstituted azulene. Enhanced activity for **7** was evident at 10^{-6} M (100% inhibition of transformation vs 87% for **13**). Moreover, **7** also increased gap junctional communication to levels comparable to that of TTNPB. However, compound **7** was slightly less active than **13** at 10^{-8} M. Like **13**, retinoid **5** contained the parent azulene end group and was a reasonably potent inhibitor. However, the presence of fluorine in **5** could also account for its activity. Once again, in agreement with results for TTNPB and its demethyl analogs, the presence of alkyl residues on the nonpolar portion of the retinoid appears to enhance activity.[31]

While fluorination did not abolish activity in **5**, the opposite was observed for the trifluoromethylated retinoid

Table I. Biological Testing of Azulenic Retinoids

			transformation assay				communication assay	
compound[a]	concn, M	toxicity % control[b]	foci/dish[c]	% control	no. dishes[d]	dishes with/ dishes without foci	no. communicating cells[e,h]	connexin43 expression[f]
acetone control		(24.9)	0		9	0/9	4.7 ± 3	ND
MCA control		100 (24.8)	1.05 ± 0.5	100	34	22/12	ND	ND
TTNPB	10^{-6}	80	0	0	12	0/12	21.2 ± 11***	+++
	10^{-8}	83	0	0	8	0/8		
	10^{-10}	95	0	0	11	0/11		
1	10^{-6}	89.1	0.8	76	10	4/6	9.1 ± 3***	ND
	10^{-8}	120	1.27	120	11	10/1		
	10^{-10}	116	1.08	103	12	9/3		
2	10^{-6}	89	1.18	112	11	7/4	8.8 ± 5*	ND
	10^{-8}	120	1.5	142	12	10/2		
	10^{-10}	125	1.5	142	12	9/3		
3	10^{-6}	125	1.25	119	12	8/4	5.7 ± 4	ND
	10^{-8}	129	1.0	95	12	8/4		
	10^{-10}	115	1.16	110	12	8/4		
7	10^{-6}	67	0	0	9	0/9	18.3 ± 7***	ND
	10^{-8}	94	0.42	40	12	5/7		
	10^{-10}	112	1.54	146	11	8/3		
8	10^{-6}	84	1.09	104	11	8/3	4.9 ± 4	ND
	10^{-8}	138	1.16	110	12	8/4		
	10^{-10}	129	1.0	95	11	8/3		
9	10^{-6}	58	0.91	87	11	5/6	8.7 ± 5**	ND
	10^{-8}	174	1.75	166	12	10/2		
	10^{-10}	142	1.5	143	12	8/4		
acetone control[g]		(21.6)	0.04		24	1/23	1.65 ± 2.6	±
MCA control[g]		100 (18.0)	0.66 ± 0.2	100	60	25/35	ND	ND
TTNPB	10^{-6}	85	0	0	12	0/12	18.5 ± 7.0***	
	10^{-8}	91	0	0	12	0/12		+++
	10^{-10}	96	0	0	12	0/12		
4	10^{-6}	178	0.5	75	12	5/7	1.55 ± 2.3	±
	10^{-8}	151	0.4	61	12	5/7		±
	10^{-10}	94	0.4	61	12	5/7		
5	10^{-6}	78	0.08	12	12	1/11	8.8 ± 8.2**	++
	10^{-8}	120	0.08	12	12	1/11		±
	10^{-10}	113	0.58	88	12	5/7		
6	10^{-6}	178	0.58	88	12	5/7	5.6 ± 7.3*	ND
	10^{-8}	156	0.58	88	12	4/8		
	10^{-10}	98	0.58	88	12	5/7		
10	10^{-6}	84	0.16	24	12	2/10	7.2 ± 8.0**	ND
	10^{-8}	129	0.16	24	12	2/10		
	10^{-10}	96	0.16	24	12	2/10		
11	10^{-6}	66	0	0	12	0/12	9.5 ± 8.0***	+++
	10^{-8}	75	0.16	24	12	2/10		+
	10^{-10}	75	0.16	24	12	2/10		
12	10^{-6}	98	0.5	75	12	5/7	2.9 ± 3.9	ND
	10^{-8}	94	0.33	50	12	4/6		
	10^{-10}	89	0.33	50	12	4/6		
13	10^{-6}	67	0.08	12	12	1/11	7.4 ± 4.9***	ND
	10^{-8}	147	0.33	50	12	2/10		
	10^{-10}	129	0.33	50	12	4/6		

[a] For structural identities, see Figure 1 and Schemes I and II. All retinoids were added as an acetone solution to cultures treated 8 days previously to 3 μg/mL MCA in acetone or to acetone alone. Total concentration of acetone was 0.5% in both cases, which is nontoxic. Treatment duration: 1 day for MCA; continous for retinoids over a 4-week period. [b] Measured as the plating efficiency for cells plated at clonal density and treated with retinoids or MCA for 7 or 1 day, respectively, as above, and normalized to the MCA-treated controls. Values in parentheses are percent plating efficiencies. [c] Numbers of type II and III morphologically transformed foci. [d] Each retinoid-treated group consisted of 12 dishes each; some dishes were lost to contamination. [e] Dye transfer measured 10 min after microinjection in cultures treated for 21 days with retinoid or acetone control. [f] As assessed by Western blotting (see Figure 2), after 7 days treatment. ND, not done. [g] Respective controls for compounds 4–6 and 10–13 tested separately from the other compounds. [h] Statistical tests: ***$P > 0.001$; **$P > 0.01$; *$P > 0.05$ compared to respective controls. Retinoid 7 was significantly more potent in induction of communication ($P > 0.05$) than other azulenic retinoids in this group.

6. Diminished activity in 6 could be a manifestation of the 2-cis configuration for the side chain since the β-CF_3 and carboxyl group have a marked preference for a trans arrangement.[23b] In this regard the reduced activity of retinoid 6 is reminiscient of 13-*cis*-retinoic acid.[31b] Alternatively, the relative polarity of the CF_3 group or its significantly increased steric bulk relative to a normal methyl could account for the loss in activity.[35]

The overall dimensions of the retinoid also appeared to influence activity. Incorporation of a second double bond in benzoic acid retinoid 12 seriously compromised its potency at 10^{-8} M relative to 7. Interestingly, the opposite effect was observed upon reduction of dosage levels to 10^{-10} M. Similar trends in activity were observed for the type I retinoids 1 vs 4. At 10^{-6} M 1 was approximately twice as potent as the longer 4, while at 10^{-10} M, the activity was reversed.

Changing the site of attachment of the ethenylbenzoic acid moiety to the guaiazulenic end group in retinoids 7, 10, and 11 not only dramatically perturbed their electronic spectra, but also resulted in altered activity. The UV–vis absorption maxima for azulenic retinoids 7, 10, and 11 are located at 428, 335, and 280 nm, respectively, which is consistent with results from other studies.[16] While both

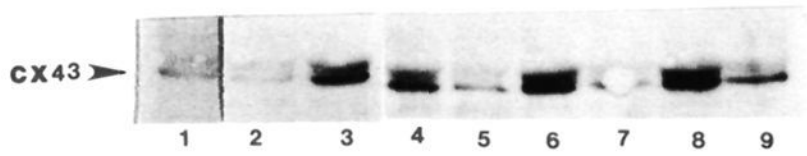


Figure 2. Azulenic retinoids up-regulate expression of connexin 43 in 10T1/2 cells. 10T1/2 cells were treated for 7 days after reaching confluence with the indicated retinoid or with acetone as control. Cells were then harvested and lysed and protein extracts subjected to electrophoresis and immunoblotting with a connexin 43 antibody as described: lane 1, acetone (0.5%) solvent control; lanes 2 and 3, retinoid **5**, 10^{-8} and 10^{-6} M; separate blot; lanes 4 and 5 retinoid **4**, 10^{-6} and 10^{-8}M; Lanes 6 and 7, retinoid **11**, 10^{-6} and 10^{-8} M; lane 8: TTNPB 10^{-8} M; lane 9, acetone solvent control.

7 (1-substituted) and **11** (8-substituted) were completely effective inhibitors at 10^{-6} M, **10** (2-substituted) was somewhat less potent (75% inhibition). Interestingly, at the reduced dose levels of 10^{-8} M all three were essentially equipotent.

The ability of retinoids to interact with putative binding sites in retinal proteins and other binding proteins or intercellular receptors is clearly affected by their overall molecular dimensions.[36] However, for the new azulenic retinoids, a second factor, namely the unique electronic properties of the nonbenzenoid aromatic moiety, might also account for the some of their activity. Azulene is highly polarizable and has a significant intrinsic dipole moment (1.09 D).[37] Its ability to stabilize positive charge by azulenylium ion formation in biological applications has been described earlier.[16] Moreover, azulenes are capable of strong hydrogen-bonding with phenols[38] and forming strong π-complexes with suitable electron-deficient acceptors.[39] It is tempting to speculate that any one or more of these unique effects might contribute to the binding interactions of azulenic retinoids with appropriate proximate amino acid residues in the recognition sites of cellular RAR (RXR) or CRABP.

Computer-Assisted Molecular Modeling. Computer-assisted molecular modeling (CAMM) of structurally modified retinoids is a valuable adjunct for the determination of retinoid activity in vision-related and anticancer applications. For the visual protein rhodopsin, energy-minimized structures for all sixteen isomers of retinal in their protonated Schiff base forms were juxtapositioned to produce a topological surface that approximated the binding site of the chromoprotein.[40] This analysis revealed regions in the binding site that were clearly inaccessible to certain geometric isomers such as the inactive 13-cis form. A similar CAMM approach was applied to bacteriorhodopsin for azulenic and C22-retinal analogs.[41] Anticancer activity has been systematically correlated for numerous modified retinoids by CAMM.[42] These rigorous semiempirical treatments have been useful as structural guidelines for the design of new retinoids.

In this study we used a CAMM approach for two of the new azulenic retinobenzoic acid analogs. The structures for **7**, **11**, and TTNPB were energy-minimized (AM-1 program) and superpositioned as shown in Figures 3 (**7** vs TTNPB) and 4 (**11** vs TTNPB). The relative differences in molecular shape for the azulenic retinoids are evident upon inspection of their structures. For both new compounds there are only slight excursions of their molecular volume from that of TTNPB. The isopropyl methyls appended to the azulene terminus protrude somewhat from this space. TTNPB is nonplanar as a result of steric interaction between the side chain methyl and benzoic acid ring. Similarly, the active retinoid **11** deviates slightly from planarity due to ring-chain steric interactions. Inspection of different views (data not shown) of these molecular models clearly reveals the similarities in spatial requirements between the new nonbenzenoid retinoids and the prototype arotinoid TTNPB. At present we cannot detect any structural features that could account for the lower activity of the new compounds.

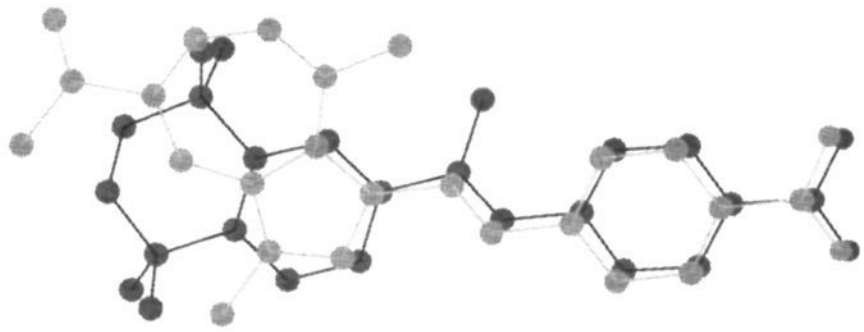


Figure 3. 1-Substituted azulenic retinoid **7** (in gray) juxtapositioned on TTNPB (in black).

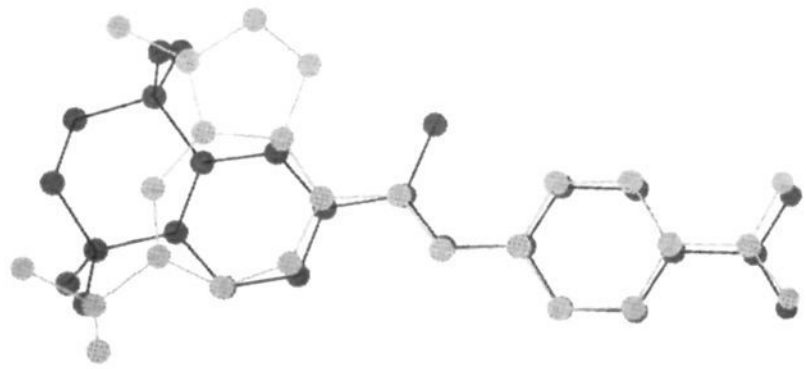


Figure 4. 8-Substituted azulenic retinoid **11** (in gray) superpositioned on TTNPB (in black).

In summary, a new class of nonbenzenoid arotinoids, the azulenic retinoids, have been shown to possess anticancer activity in the very sensitive and highly reliable *in vitro* mouse fibroblast C3H10T1/2 cell assay. We anticipate that further systematic structural modifications of these novel retinoids will not only lead to enhanced inhibitory activity but also contribute to the clarification of the molecular basis of this activity.

Experimental Section

General. Infrared spectra were obtained on a Perkin-Elmer 1600 Series FT-IR instrument. Ultraviolet spectra were recorded on a Perkin-Elmer Lambda-19 spectrophotometer. For compounds with several absorption maxima, the largest peak is *italicized*. ^{1}H-NMR spectra were recorded on a General Electric LQE-300 (300 MHz) or GN Omega 500 (500 MHz) FT-NMR spectrometer. Chemical shifts are reported in δ units (ppm) relative to tetramethylsilane as internal standard. ^{19}F-NMR spectra were recorded on a Nicolet NT-300 spectrometer at 283 MHz with fluorotrichloromethane as the internal reference. Mass spectra were obtained at 70 eV in the electron-impact mode on a VG Analytical 70-SE high-resolution dual-focusing mass spectrometer with an 11-250 data system. Melting points were determined on a Laboratory Devices Mel-Temp II melting point apparatus and are uncorrected.

Anhydrous THF was freshly distilled from benzophenone ketyl. Solutions of lithium diisopropylamide (LDA) were prepared from the reaction of 1 equiv of butyllithium with 1.1 equiv of diisopropylamine (distilled from calcium hydride and stored over activated 4-Å molecular sieves) in THF at −40 °C for 15 min. Product yields were not optimized. Column chromatographic separations were performed using Fisherbrand 100–200 mesh silica gel for open-column applications or ICN Associates silica gel 32–63 mesh, 60A for flash column chromatography. Thin-layer chromatographic analyses were performed on Sigma precoated silica gel 60-254F plates.

3-[1-[3,8-Dimethyl-5-(1-methylethyl)azulenyl]]-2-propenenitrile. To the lithium salt of (diethylphosphono)aceto-

nitrile, prepared from (diethylphosphono)acetonitrile (2.12 g, 12 mmol) and butyllithium (12.5 mmol) in THF (25 mL), was added guaiazulene-1-carboxaldehyde[21] (1.14 g, 5.0 mmol) in THF (10 mL) at −78 °C. After stirring for 1 h at room temperature, the reaction mixture was quenched with dilute citric acid and extracted with 50% ether–hexanes. The combined organic layers were washed with water followed by brine solution, dried ($MgSO_4$), filtered, and concentrated to give the crude nitrile as a dark green solid. Recrystallization from ethyl acetate–pentane afforded pure nitrile (0.70 g, 56% yield) as dark green needles: mp 104–106 °C; IR (film) 2950, 2201 (CN), 1580, 1548, 1415, 1366, 1192, 954, 648 cm^{-1}; UV (ethanol) 332, *414*, 585 nm; ^1H NMR δ 1.36 (6H, d, 7.0 Hz), 2.58 (3H, s, C3′-CH_3), 3.00 (3H, s, C8′-CH_3), 3.07 (1H, m, 7.0 Hz), 5.60 (1H, d, 15.9 Hz, =C*H*CN), 7.12 (1H, d, 10.7 Hz, C7′-H), 7.42 (1H, dd, 2 and 10.5 Hz, C6′-H), 7.78 (1H, s, C2′-H), 8.12 (1H, d, 2 Hz, C4′-H), 8.29 (1H, d, 15.9 Hz, C*H*=CHCN) ppm. **3-[1-[3,8-Dimethyl-5-(1-methylethyl)azulenyl]]-2-propenal (14).** Reduction of the above nitrile (0.70 g, 2.7 mmol) with DIBAL solution (3 mL, 3 mmol) in ether–pentane–dichloromethane (7:3:1) at −78 °C for 1 h followed by wet silica gel workup gave the corresponding aldehyde 14 (0.49 g) as a dark green-black solid in 72% yield. Recrystallization from ether–hexanes afforded pure 14 as glistening black platelets: mp 124–125 °C (lit.[21] mp 125 °C); IR (film) 2797, 2704, 1659 (C=O), 1135 cm^{-1}; UV (ethanol) 341, *438*, *ca.* 580 nm; ^1H NMR δ 1.36 (6H, d, 6.9 Hz), 2.55 (3H, s, C3′-CH_3), 3.00 (3H, s, C8′-CH_3), 3.07 (1H, m), 6.57 (1H, dd, 8.0 and 15.0 Hz, =C*H*CHO), 7.12 (1H, d, 10.7 Hz, C7′-H), 7.42 (1H, dd, 1.9 and 10.7 Hz, C6′-H), 7.83 (1H, s, C2′-H), 8.11 (1H, d, 2.0 Hz, C4′-H), 8.31 (1H, d, 15.0 Hz, C*H*=), 9.60 (1H, d, 8.0 Hz, CHO) ppm.

Methyl 7-[1-[3,8-Dimethyl-5-(1-methylethyl)azulenyl]]-3-methyl-2,4,6-heptatrienoate (16). To a stirred, cold (−78 °C) solution of LDA (15 mmol) in THF (20 mL) was added a solution of methyl 4-(diethylphosphono)-3-methyl-2-butenoate (15) (2.75 g, 11 mmol)) in THF (20 mL). After 30 min a solution of enal 14 (1.97 g, 7.8 mmol) in THF (10 mL) was added and the reaction mixture stirred at room temperature for 2 h. After workup by quenching with dilute citric acid and extraction with ether, the dark crude product was column chromatographed using 15% ether–10% dichloromethane–75% pentane to give the desired methyl heptatrienoate 16 (2.28 g) as a dark solid in 84% yield. Recrystallization of a portion from ethyl acetate–pentane gave pure trienoate 16 (0.852 g) as purple platelets: mp 132–133 °C; IR (film) 1708 (C=O), 1566, 1144 cm^{-1}; UV (ethanol) 346, *457*, 647 nm; ^1H NMR δ 1.33 (6H, d, 7.0 Hz), 2.38 (3H, s, C3-CH_3), 2.58 (3H, s, C3′-CH_3), 2.99 (3H, s, C8′-CH_3), 3.0 (1H, m), 3.72 (3H, s, OCH_3), 5.78 (1H, s, C2-H), 6.33 (1H, d, 14.7 Hz, C4-H), 6.77 (1H, dd, 10.6 and 15 Hz, C6-H), 6.87 (1H, d, 10.4 Hz, C7′-H), 6.88 (1H, dd, 10.8 and 14.6 Hz, C5-H), 7.23 (1H, dd, 2.0 and 11 Hz, C6′-H), 7.65 (1H, d, 14.7 Hz, C7-H), 7.86 (1H, s, C2′-H), 7.96 (1H, d, 2.0 Hz, C4′-H) ppm.

7-[1-[3,8-Dimethyl-5-(1-methylethyl)azulenyl]]-3-methyl-2,4,6-heptatrienoic Acid (1). Saponification of ester 16 (0.9 g) using potassium hydroxide (5 g) in 5% aqueous methanol (45 mL) in the presence of 18-crown-6 (0.1 g) at gentle reflux for 2 h afforded crude guaiazulenic trienoic acid 1 after acidification with citric acid and extraction with ether. Recrystallization from ethyl acetate–pentane gave pure trienoic acid 1 (0.403 g) as a brown solid in 47% yield: mp 186–188 °C; IR (film) 3200–2300 (OH), 1667 (C=O) cm^{-1}; UV (ethanol) 339, *434*, 632 nm; ^1H NMR (acetone-d_6) δ 1.32 (6H, d, 7 Hz), 2.35 (3H, s, C3-CH_3), 2.57 (3H, s, C3′-CH_3), 2.99 (3H, s, C8′-CH_3), 3.05 (1H, m, 7 Hz), 5.81 (1H, s, C2-H), 6.44 (1H, d, 14.9 Hz, C4-H), 6.90 (1H, dd, 10.9 and 14.8 Hz, C6-H), 6.94 (1H, d, 10.6 Hz, C7′-H), 7.06 (1H, dd, 10.9 and 15 Hz, C5-H), 7.34 (1H, dd, 1.9 and 10.5 Hz, C6′-H), 7.80 (1H, d, 14.7 Hz, C7-H), 7.95 (1H, s, C2′-H), 8.05 (1H, d, 1.9 Hz, C4′-H) ppm; exact mass (EI) calcd for $C_{23}H_{26}O_2$ (M^+) 334.1933, found 334.1936.

7-[1-[3,8-Dimethyl-5-(1-methylethyl)azulenyl]]-3-methyl-2,4,6-heptatrien-1-ol (2). A solution of DIBAL (6 mmol) was added to a stirred, cold (−78 °C) solution of triene ester 16 (0.429 g, 1.23 mmol) in 1:1 ether–pentane (50 mL). After 30 min the reaction was quenched with wet silica gel, filtered, and concentrated to give trienol 2 (0.334 g) as a dark solid in 85% yield. Recrystallization from ether–hexanes gave pure 2 as dark green platelets: mp 126–128 °C dec; IR (film) 3600–3100 (OH); UV (ethanol) 324, *335*, 417, 654 nm; ^1H NMR δ 1.33 (6H, d, 7 Hz), 1.90 (3H, s, C3-CH_3), 2.59 (3H, s, C3′-CH_3), 2.98 (3H, s, C8′-CH_3), 3.0 (1H, m), 4.33 (2H, d, 6.0 Hz, CH_2OH), 5.71 (1H, t, 6.9 Hz, C2-H), 6.34 (1H, d, 15.1 Hz, C4-H), 6.53 (1H, dd, 10.5 and 15.1 Hz, C6-H), 6.73 (1H, dd, 10.5 and 15.0 Hz, C5-H), 6.8 (1H, d, 10.9 Hz, C7′-H), 7.19 (1H, dd, 1.8 and 10.6 Hz, C6′-H), 7.50 (1H, d, 15.0 Hz, C7-H), 7.84 (1H, s, C2′-H), 7.94 (1H, d, 2 Hz, C4′-H) ppm, exact mass (EI) calcd for $C_{23}H_{28}O$ (M^+) 320.2141, found 320.2148.

7-[1-[3,8-Dimethyl-5-(1-methylethyl)azulenyl]]-3-methyl-2,4,6-heptatrienonitrile (18) and 7-[1-[3,8-Dimethyl-5-(1-methylethyl)azulenyl]]-3-methyl-2,4,6-heptatrienal (3). To a stirred, cold (−78 °C) solution of LDA (7.0 mmol) in THF (10 mL)) was added 4-(diethylphosphono)-3-methyl-2-butenonitrile (17) (1.52 g, 7.0 mmol) in THF (15 mL) followed after 10 min by a solution of enal 14 (0.95 g, 3.8 mmol) in THF (20 mL). The reaction mixture was stirred at room temperature for 1 h and worked up as described above. The crude product was column chromatographed using ether–dichloromethane–pentane (1:1:2) to give trienenitrile 18 (2-cis:2-trans = 1:10) which was used immediately in the following conversion to trienal 3.

To a solution of partially purified trienenitrile 18 (ca. 1 g) in dichloromethane–ether (25 mL) at −78 °C was added DIBAL solution (10 mmol). The reaction mixture was stirred at room temperature for 1 h to give the corresponding crude aldehyde after the usual work up with wet silica gel. Column chromatography using 20% ether–pentane gave a mixture of 2-*cis*- and 2-*trans*-heptatrienal 3 free of contaminants. A small amount of pure all-trans trienal (70 mg) was subsequently obtained by recrystallization from ethyl acetate–hexanes: IR (film) 1654, 1579, 1416, 1157 cm^{-1}; UV (ethanol) 352, 479 nm; ^1H NMR δ 1.33 (6H, d, 7 Hz), 2.29 (3H, s C3-CH_3), 2.58 (3H, s, C3′-CH_3), 3.00 (3H, s, C8′-CH_3), 3.0 (1H, m), 5.91 (1H, d, 8 Hz, C2-H), 6.66 (1H, d, 15 Hz, C4-H), 6.86 (1H, dd, 11 and 15 Hz, C6-H), 6.97 (1H, d, 10 Hz, C7′-H), 7.05 (1H, dd, 11 and 15 Hz, C5-H), 7.33 (1H, d, 10 Hz, C6′-H), 7.77 (1H, d, 15 Hz, C7-H), 7.90 (1H, s, C2′-H), 8.00 (1H, s, C4′-H), 10.10 (1H, d, 8 Hz, CHO) ppm; exact mass (EI) calcd for $C_{23}H_{26}O$ (M^+) 318.1984, found 318.2002.

5-[1-[3,8-Dimethyl-5-(1-methylethyl)azulenyl]]-3-methyl-2,4-pentadienenitrile (19). To a stirred, cold (−78 °C) solution of LDA (10 mmol) in THF (10 mL) was added a solution of C_5-phosphononitrile 17 (2.17 g, 6.55 mmol) in THF (10 mL). After 10 min a solution of guaiazulene-1-carboxaldehyde (1.48 g, 6.5 mmol) in THF (10 mL) was added and the reaction mixture stirred at room temperature for 2 h. After quenching with dilute citric acid and extraction with ether–hexanes (1:1), filtration through a pad of silica gel using 20% ether–hexanes afforded the title dienenitrile 19 (1.05 g) in 58.3% yield as a dark green solid: mp 119–120 °C; IR (film) 2200, 1584, 1147, 949 cm^{-1}; UV (ethanol) 338, *439*, 608 nm; ^1H NMR δ 1.34 (6H, d, 6.9 Hz), 2.30 (3H, s, C3-CH_3), 2.59 (3H, s, C3′-CH_3), 3.00 (3H, s, C8′-H), 3.02 (1H, m), 5.21 (1H, s, C2-H), 6.72 (1H, d, 15.5 Hz, C4-H), 6.97 (1H, d, 10.6 Hz, C7′-H), 7.32 (1H, dd, 1.9 and 10.6 Hz, C6′-H), 7.85 (1H, s C2′-H), 7.87 (1H, s, 15.0 Hz, C5-H), 8.04 (1H, d, 2.0 Hz, C4′-H) ppm.

5-[1-[3,8-Dimethyl-5-(1-methylethyl)azulenyl]]-3-methyl-2,4-pentadienal (20). Reduction of nitrile 19 (1.05 g, 3.6 mmol) in ether (45 mL)–dichloromethane (5 mL) at −78 °C with DIBAL solution (6 mmol) for 45 min, followed by quenching with wet silica gel and subsequent column chromatography on silica gel using 33% ether–hexane gave dienal 20 (0.566 g) as brick red needles in 54% yield: mp 111–112 °C; IR (film) 1652, 1574, 1331, 1205, 1130 cm^{-1}; UV (ethanol) 340, *465* nm; ^1H NMR δ 1.35 (6H, d, 7 Hz), 2.41 (3H, s, C3-CH_3), 2.60 (3H, s, C3′-CH_3), 3.04 (3H, s, C8′-CH_3), 3.04 (1H, m), 6.07 (1H, d, 8.2 Hz, C2-H), 6.80 (1H, d, 15.5 Hz, C4-H), 6.99 (1H, d, 10.6 Hz, C7′-H), 7.32 (1H, d, 10.7 Hz, C6′-H), 7.92 (1H, s, C2′-H), 8.04 (1H, s, C4′-H), 8.07 (1H, d, 15.5 Hz, C5-H), 10.13 (1H, d, 8 Hz, CHO) ppm.

3,7-Dimethyl-9-[1-[3,8-dimethyl-5-(1-methylethyl)azulenyl]]-2,4,6,8-nonatetraenoic Acid (4). To a solution of LDA (10 mmol) in THF (10 mL) was added a solution of C_5-phosphono ester 15 (2.5 g, 10 mmol) in THF (10 mL) at −78 °C. After 15 min dienal 20 (1.07 g, 3.66 mmol) in THF (10 mL) was added and the reaction mixture stirred at room temperature for 1 h. After workup by acidification with dilute citric acid and extraction with 1:1 ether–hexanes followed by column chromatography using

8% ether–hexanes, the tetraene ester precursor of 4 was obtained as a dark solid (0.89 g) in 63% yield. A portion of this ester (0.59 g, 1.52 mmol) was hydrolyzed with excess KOH (2.5 g, 45 mmol) in 15% aqueous methanol (35 mL) by gently refluxing for 1.5 h. Upon acidification with dilute citric acid solution a dark red solid precipitated and was collected by filtration. Recrystallization from aqueous methanol afforded the desired tetraenoic acid 4 (0.38 g) as a brick red solid in 67% yield: mp 150–152 °C; IR (film) 3200–2000 (OH), 1672 (C=O) cm^{-1}; UV (ethanol) 351, *443*, 654 nm; ^1H NMR δ 1.33 (6H, d, 7 Hz), 1.6 (1H, bs, CO_2H), 1.12 (3H, s, C7-CH_3), 2.39 (3H, s, C3-CH_3), 2.59 (3H, s, C3′-CH_3), 2.95 (1H, m), 3.00 (3H, s, C8′-CH_3), 5.82 (1H, s, C2-H), 6.32 (1H, d, 11.4 Hz, C6-H), 6.35 (1H, d, 14.7 Hz, C4-H), 6.78 (1H, d, 15.2 Hz, C8-H), 6.83 (1H, d, 11.7 Hz, C7′-H), 7.10 (1H, dd, 11.6 and 14.9 Hz, C5-H), 7.22 (1H, dd, 1.5 and 10.5 Hz, C6′-H), 7.65 (1H, d, 15.4 Hz, C9-H), 7.86 (1H, s, C2′-H), 7.95 (1H, d, 1.8 Hz, C4′-H) ppm; exact mass (EI) calcd for $C_{26}H_{30}O_2$ (M^+) 374.2246, found 374.2247.

3-(1-Azulenyl)propenenitrile and 3-(1-Azulenyl)propenal. To a solution of lithium diethyl (cyanomethyl)phosphonate (3.0 g, 17 mmol) in THF (20 mL) was added a solution of azulene-1-carboxaldehyde[21] (0.78 g, 5.0 mmol) in THF (15 mL) at −78 °C. After stirring at room temperature for 45 min the reaction mixture was worked up in the usual manner to afford the desired nitrile (0.72 g) in 80% yield as a blue-gray solid.

Reduction of this nitrile (0.72 g, 4.0 mmol) with excess DIBAL (8 mmol) for 1 h at −78 °C followed by wet silica gel workup and column chromatography using 25% ether–hexanes gave 3-(1-azulenyl)propenal[22] (0.615 g) as a dark green solid in 84% yield; ^1H NMR δ 6.77 (1H, dd, 7.8 and 15.3 Hz, C2-H), 7.2–7.9 (4H, m), 8.07 (1H, d, 16 Hz, C3-H), 8.20 (1H, d, 4.6 Hz), 8.35 (1H, d, 9 Hz), 8.58 (1H, d, 9.6 Hz), 9.74 (1H, d, 7.8 Hz, CHO) ppm.

Ethyl 7-(1-Azulenyl)-2-fluoro-3-methyl-2,4,6-heptatrienoate (22) and 7-(1-Azulenyl)-2-fluoro-3-methyl-2,4,6-heptatrienoic Acid (5). To a stirred, cold (−78 °C) solution of LDA (5 mmol) in THF (10 mL) was added a solution of ethyl 4-(diethylphosphono)-2-fluoro-3-methyl-2-butenoate (21) (1.5 g, 5.3 mmol) in THF (8 mL) to give a clear red solution of the anion. After 10 min a solution of 3-(1-azulenyl)propenal (0.615 g, 3.38 mmol) in THF (10 mL) was added and the reaction mixture stirred at room temperature for 2 h. Workup in the usual manner as described above afforded crude azulenic fluoro ester 22 as a dark red solid. Column chromatographic purification using 15% ether–hexanes gave heptatrienoate 22 (0.604 g) as a dark brown solid in 58% isolated yield: mp 101–103 °C dec; IR (film) 1707 cm^{-1}; UV (ethanol) 334, *430*, 633 nm; ^1H NMR δ 1.37 (3H, t, 7 Hz), 2.085 (d, 4.5 Hz, C3-CH_3 for 2-cis isomer), 2.30 (d, 3.2 Hz, C3-CH_3 for 2-trans isomer), 6.85–8.45 (11H, m) ppm; 2-cis:2-trans = 2:1.

Hydrolysis of the fluoro ester 22 (224 mg, 0.72 mmol) with aqueous methanolic KOH (gentle reflux, 1 h) gave the corresponding fluoro acid 5 (190 mg) as a brown solid in 93% yield: mp 88 °C dec; IR (film) 1682 (C=O); UV (ethanol) 329, *411*, 630 nm; ^1H NMR (acetone-d_6) for major 2*Z*-(2-trans) isomer δ 2.29 (3H, d, 3.2 Hz, C3-CH_3), 6.85 (1H, 15.3 Hz, C4-H), 7.03 (1H, dd, 11 and 15.8 Hz, C6-H), 6.8–8.6 (9H, m); ^{19}F NMR (CD_2Cl_2) δ −125.7 ppm; for minor 2*E*-(2-cis) isomer of 5; ^1H NMR (acetone-d_6) δ 2.05 (3H, d, 4.5 Hz, C3-CH_3) ppm; ^{19}F NMR (CD_2Cl_2) δ −122.0 ppm; exact mass (EI) calcd for $C_{18}H_{15}FO_2$ (M^+) 282.1056, found 282.1067.

6-[1-[3,8-Dimethyl-5-(1-methylethyl)azulenyl]]-1,1,1-trifluoro-3,5-hexadien-2-one (23) and 7-[1-[3,8-Dimethyl-5-(1-methylethyl)azulenyl]]-3-(trifluoromethyl)-2,4,6-heptatrienoic Acid (6). The piperidinium acetate-catalyzed condensation[23] of guaiazulenic enal 14 with excess trifluoroacetone in THF afforded the unsaturated trifluoromethyl ketone 23 as the sole product. Treatment of ketone 23 (0.53 g, 1.54 mmol) with the sodium salt of triethyl phosphonoacetate (1.34 g, 6 mmol) in THF (25 mL) for 4 h at room temperature gave the desired fluorinated trienoate after normal workup: IR (film) 1712 cm^{-1}.

Base hydrolysis of the ester (0.5 g, 1.2 mmol) with excess KOH (2 g, 36 mmol) in 80% aqueous methanol (30 mL) and THF (10 mL) at gentle reflux for 1 h followed by acidification with dilute citric acid solution gave a black precipitate (0.27 g) of trifluoromethyl carboxylic acid 6 in 65% yield: mp 146–148 °C; IR (film) 2100–3400 (br, OH), 1683 (C=O) cm^{-1}; UV (ethanol) 337, *433*, 620 nm; ^1H NMR δ 1.33 (6H, d, 7 Hz), 1.7 (bs, CO_2H), 2.58 (3H, s, C3′-CH_3), 3.0 (1H, m), 3.02 (3H, s, C8′-CH_3), 6.13 (1H, s, C2-H), 6.88 (1H, dd, 11 and 14.5 Hz, C6-H), 6.94 (1H, d, 10.3 Hz, C7′-H), 7.09 (1H, dd, 11 and 16 Hz, C5-H), 7.28 (1H, d, 10 Hz, C6′-H), 7.56 (1H, d, 15.8 Hz, C4-H), 7.78 (1H, d, 14.8 Hz, C7-H), 7.92 (1H, s, C2′-H), 8.01 (1H, d, 1 Hz, C4′-H) ppm; ^{19}F NMR (CD_2Cl_2) δ −63.6 ppm; exact mass (EI) calcd for $C_{23}H_{23}F_3O_2$ (M^+) 388.16501, found 388.1666.

Methyl 4-[2-[1-[3,8-Dimethyl-5-(1-methylethyl)-azulenyl]]ethenyl]benzoate (25). To a stirred, cooled (−78°) solution of LDA (13 mmol) was added a solution of methyl 4-[(diethylphosphono)methyl]benzoate 24[24] (3.72 g, 13 mmol) in THF (10 mL) to give a deep red solution of the lithium salt. After 10 min a solution of guaiazulene-1-carboxaldehyde (2.26 g, 10 mmol) in THF (15 mL) was added and the reaction mixture stirred at room temperature for 2.5 h. After conventional workup, guaiazulenic benzoate ester 25 was obtained as a dark green solid. Recrystallization from ethyl acetate–hexane afforded 1.83 g of pure methyl ester 25 as dark green needles in 51% yield; mp 130–131 °C; IR (film) 1716 cm^{-1}; UV (ethanol) 279, 337, *434*, 624 nm; ^1H NMR δ 1.37 (6H, d, 7.0 Hz), 2.64 (3H, s, C3′-CH_3), 3.02 (1H, m), 3.09 (3H, s, C8′-CH_3), 3.94 (3H, s, OCH_3), 6.93 (1H, d, 10.6 Hz, C7′-H), 6.96 (1H, d, 15.8 Hz, vinyl H), 7.3 (1H, dd, 1.8 and 10 Hz, C6′-H), 7.55 (2H, d, 8.4 Hz, phenyl H), 7.96 (1H, s, C2′-H), 8.03 (2H, d, 8.2 Hz, phenyl H), 8.04 (1H, s, C4′-H), 8.18 (1H, d, 15.8 Hz, vinyl H) ppm.

4-[2-[1-[3,8-Dimethyl-5-(1-methylethyl)azulenyl]]ethenyl]-benzoic Acid (7). Ester 25 (0.2 g, 0.56 mmol) was gently refluxed with excess KOH (0.5 g, 8.9 mmol) in methanol (5 mL) for 3 h. After acidification with citric acid and ether extraction of the crude acid, recrystallization from ethyl acetate–hexanes gave pure guaiazulenic benzoic acid 7 (0.108 g) as dark green needles in 56% yield: mp 260–4 °C dec; IR (film) 1676 cm^{-1} (C=O); UV (ethanol) 276, 333, *428*, 632 nm; ^1H NMR (acetone-d_6) δ 1.36 (6H, d, 7 Hz), 1.8 (1H, bs, CO_2H), 2.63 (3H, s, C3′-CH_3), 3.03 (1H, m), 3.08 (3H, s, C8′-CH_3), 6.93 (1H, d, 10 Hz, C7′-H), 6.97 (1H, d, 14.4 Hz, vinyl-H), 7.29 (1H, d, 11.6 Hz, C6′-H), 7.57 (2H, d, 8.2 Hz, phenyl-H), 7.96 (1H, s, C2′-H), 8.04 (1H, s, C4′-H), 8.07 (2H, d, 8.3 Hz, phenyl-H), 8.29 (1H, d, 15.8 Hz, vinyl-H) ppm; exact mass (EI) calcd for $C_{24}H_{24}O_2$ (M^+) 344.1776, found 344.1802.

4-[2-[1-[3,8-Dimethyl-5-(1-methylethyl)azulenyl]]ethenyl]-benzyl Alcohol (8). Reduction of guaiazulenic benzoate 25 (0.249 g, 0.7 mmol) in ether (20 mL) and dichloromethane (5 mL) at −78 °C for 1 h using an excess of DIBAL (5 mmol) followed by wet silica gel workup gave the desired guaiazulenic benzyl alcohol 8 (0.177 g), an emerald green solid in 76% yield after recrystallization from ether–hexane: mp 126–127 °C; IR (film) 3357 (bs, OH); UV (ethanol) 266, *327*, 411, 642 nm; ^1H NMR δ 1.39 (6H, d, 7 Hz), 2.67 (3H, s, C3′-CH_3), 3.06 (1H, m), 3.10 (3H, s, C8′-CH_3), 4.75 (2H, d, 6 Hz, CH_2OH), 6.91 (1H, d, 10.6 Hz, C7′-H), 6.97 (1H, d, 15.9 Hz, vinyl H), 7.29 (1H, dd, 1.6 and 10 Hz, C6′-H), 7.40 (2H, d, 8.1 Hz, phenyl H), 7.55 (2H, d, 8.1 Hz phenyl H), 7.97 (1H, s, C2′-H), 8.05 (1H, s, C4′-H), 8.08 (1H, d, 16.1 Hz, vinyl H) ppm; exact mass (EI) calcd for $C_{24}H_{26}O$ (M^+) 330.1984, found 330.1990.

4-[2-[1-[3,8-Dimethyl-5-(1-methylethyl)azulenyl]]ethenyl]-benzonitrile (27) and 4-[2-[1-[3,8-Dimethyl-5-(1-methylethyl)azulenyl]]ethenyl]benzaldehyde (9). To a stirred, cooled (−78 °C) solution of LDA (1.25 mmol) was added a solution of 4-[(diethylphosphono)methyl]benzonitrile (26) (0.3 g, 1.2 mmol) in THF (10 mL) over 2 min. After an additional 30 min a THF (10 mL) solution of guaiazulene-1-carboxaldehyde (0.2 g, 0.88 mmol) was added and the mixture stirred for 4 h at room temperature. After the usual workup nitrile 27 was obtained as a dark viscous oil (0.1 g) which was purified by filtration through a short column of silica gel using 20% ethyl acetate–hexane to remove polar impurities.

A solution of DIBAL (2 mmol) was added over 1 min to a stirred, cooled (−78 °C) solution of benzonitrile 27 (0.1 g, 0.31 mmol) in THF (15 mL). After 1 h at room temperature the reaction mixture was quenched with an ether slurry of wet silica gel, filtered, and column chromatographed using 20% ethyl acetate–hexanes to give pure guaiazulenic benzaldehyde analog 9 (70 mg) as dark green, flat needles in 69% yield: mp 128–129 °C; IR (film) 1692 cm^{-1}; UV (ethanol) 284, 335, *449*, 620 nm; ^1H NMR δ 1.38 (6H, d, 7.0 Hz), 2.66 (3H, s, C3′-CH_3), 3.0 (1H, m),

3.10 (3H, s, C8′-CH_3), 6.97 (1H, d, 10.6 Hz, C7′-H), 6.99 (1H, d, 15.8 Hz, vinyl H), 7.32 (1H, dd, 1.6 and 10.6 Hz, C6′-H), 7.64 (2H, d, 8.2 Hz, phenyl H), 7.87 (2H, d, 8.2 Hz, phenyl H), 7.98 (1H, s, C2′-H), 8.06 (1H, d, 1.9 Hz, C4′-H), 8.24 (1H, d, 15.8 Hz, vinyl H), 9.99 (1H, s, CHO) ppm; exact mass (EI) calcd for $C_{24}H_{24}O$ (M^+) 328.1827, found 328.1816.

Guaiazulene-2-carboxaldehyde (28). The thermal rearrangement of guaiazulene-1-carboxaldehyde to the corresponding isomeric 2-carboxaldehyde was carried out by a modified procedure of Kurokawa.[25] Guaiazulene-1-carboxaldehyde (1.01 g, 4.5 mmol) was heated for 90 s in a test tube on a micro Bunsen burner. A blue condensate formed and the mixture darkened. After cooling to room temperature, the mixture was triturated with ether (10 mL) and filtered and the intractable dark residue washed free of colored material with additional ether (50 mL). The filtrate was then concentrated and the crude product column chromatographed using 10% ether–hexanes. An initial blue band of guaiazulene and other nonpolar products was followed successively by an unidentified yellow-brown component, an emerald green component, and, lastly, a red-brown band of recovered starting material. The emerald green band was collected and the solvent removed to give the desired 2-carboxaldehyde **28** (81 mg) as a dark green oil in 8.0% isolated yield: ^{1}H NMR δ 1.42 (6H, d, 7 Hz), 2.88, 2.94 (each 3H and s, C3′- and C8′-CH_3), 3.12 (1H, m), 7.08 (1H, d, 10.5 Hz, C7′-H), 7.54 (1H, dd, 1.5 and 10.5 Hz, C6′-H), 7.66 (1H, s, C1′-H), 8.43 (1H, d, 1.5 Hz, C4′-H), 10.64 (1H, s, CHO) ppm.

Methyl 4-[2-[2-[3,8-Dimethyl-5-(1-methylethyl)azulenyl]]ethenyl]benzoate and 4-[2-[2-[3,8-Dimethyl-5-(1-methylethyl)azulenyl]]ethenyl]benzoic Acid (10). To a stirred, cold (−78 °C) solution of LDA (0.74 mmol) in THF (5 mL) was added a solution of phosphonate **24** (0.224 g, 0.74 mmol) in THF (5 mL). After 15 min a solution of aldehyde **28** (0.084 g, 0.37 mmol) in THF (5 mL) was added and the reaction mixture stirred at room temperature for 45 min. After normal workup and column chromatography using 8% ether–hexanes the desired methyl ester (0.124 g) was obtained as a dark green solid in 93% yield: mp 131.5–134 °C; IR (film) 1716 (C=O) cm^{-1}; UV (ethanol) *333*, 387, 406, 428, 613, 659 nm; ^{1}H NMR δ 1.36 (6H, d, 7 Hz), 2.70 (3H, s, C2′- or C8′-CH_3), 2.83 (3H, s, C2′- or C8′-CH_3), 3.05 (1H, m), 3.93 (3H, s, OCH_3), 6.98 (1H, d, 10.5 Hz, C7′-H), 7.30 (1H, d, 10.5 Hz, C6′-H),7.37 (2H, d, 7.9 Hz, phenyl H), 7.46 (1H, d, 16.4 Hz, vinyl H), 7.67 (1H, d, 16.1 Hz, vinyl H), 8.05 (2H, d, 7.9 Hz, phenyl H), 8.12 (1H, s, C4′-H) ppm.

Acid **10** was prepared from the above ester (0.085 g, 0.24 mmol) in the same a manner as benzoic acid analog **7**. Recrystallization from ethyl acetate–hexanes gave pure **10** (73 mg) as dark green needles in 89% yield: mp 158–160 °C; IR (film) 1681 (C=O), 1601, 1425, 1291, 1176, 954 cm^{-1}; UV (ethanol) *335*, 390, 407, 429, 648 nm; ^{1}H NMR ($CDCl_3$) δ 1.34 (6H, d, 7 Hz), 2.69 (3H, s, C3′-CH_3), 2.81 (3H, s, C8′-CH_3), 3.04 (1H, m, 7 Hz), 6.97 (1H, d, 10.5 Hz, C7′-H), 7.29 (1H, d, 10.5, C6′-H), 7.40 (1H, s, C1′-H), 7.40 (1H, d, 16.2 Hz, vinyl H),7.68 (2H, d, 9 Hz, phenyl H), 8.10 (2H, d, 8.0 Hz, phenyl H), 8.11 (1H, s, C4′-H) ppm; exact mass (EI) calcd for $C_{24}H_{24}O_2$ (M^+) 344.1776, found 344.1781.

4-[2-[8-[3-Methyl-5-(1-methylethyl)azulenyl]]ethenyl]benzoic Acid (11). To a solution of LDA (18 mmol) was added a solution of guaiazulene (2.9 g, 14.6 mmol) in THF (25 mL) at −78 °C to give a dark red solution of lithioguaiazulenide. After 10 min, a solution of methyl 4-formylbenzoate (2.4 g, 14.6 mmol) in THF (10 mL) was added and the reaction mixture reverted back to a blue color. After stirring for 2.5 h at room temperature, the reaction was worked up in the usual manner. Column chromatographic purification using 15–40% ether–hexanes afforded hydroxy ester **29** as a blue oil (3.46 g) in 66% yield. Recrystallization from ether–pentane gave pure **29** as a blue solid: mp 86.0–86.5 °C; IR (film) 3479 (OH), 2957, 1721 (ester C=O), 1611, 1555, 1436, 1280, 1112, 1019, 761 cm^{-1}; UV (ethanol) *286*, 338, 351, 368, 612 nm; ^{1}H NMR δ 1.38 (6H, d, 7 Hz), 1.7 (1H, bs, OH), 2.69 (1H, s, C3′-CH_3), 3.10 (1H, m), 3.42 (1H, dd, 9.4 and 13.2 Hz, C8′-CH_AH_B), 3.56 (1H, dd, 3.8 and 14.4 Hz, C8′-CH_AH_B), 3.94 (3H, s, OCH_3), 5.24 (1H, dd, 3.6 and 9.2 Hz, C*H*OH), 7.01 (1H, d, 10.8 Hz, C7′-H), 7.26 (1H, bs), 7.44 (1H, dd, 1.9 and 10.8 Hz, C6′-H), 7.71 (1H, s), 8.06 (2H, d, 8.3 Hz, phenyl H), 8.24 (1H, d, 1.9 Hz, C4′-H) ppm.

To a solution of hydroxy ester (1.5 g, 4.1 mmol) in benzene (10 mL) was slowly added a large excess of phosphoryl chloride (10 mL) at room temperature. After the initial reaction subsided, the mixture was heated to gentle reflux for 1 h. Excess pyridine (10 mL) was then added and the mixture refluxed for an additional 1 h. After cooling the solution was neutralized with dilute HCl and extracted with ether–hexanes. The resulting crude ester mixture was then hydrolyzed by gently refluxing with aqueous methanoic KOH (2.4 g, 43 mmol) for 1 h. After the usual workup, the crude acid was recrystallized from benzene–hexanes to give pure acid **11** (0.45 g) as a dark green solid in 33% yield: mp 228–230 °C; IR (film) 1679, 1602, 1420, 1289 cm^{-1}; UV (ethanol) *280*, 320, 348 sh, 645 nm; ^{1}H NMR (acetone-d_6) δ 1.35 (6H, d, 7 Hz), 2.62 (3H, s, C3′-CH_3), 3.12 (1H, m 7 Hz), 7.55-7.68 (4H, m, C1′-H, C2′-H, C6′-H, and C7′-H), 7.58 (1H, d, 16.1 Hz, vinyl H), 7.89 (2H, d, 8.3 Hz, phenyl H), 8.06 (2H, d, 8.2 Hz, phenyl H), 8.23 (1H, s, C4′-H), 8.28 (1H, d, 16.2 Hz, vinyl H) ppm; exact mass (EI) calcd for $C_{23}H_{22}O_2$ (M^+) 330.1620, found 330.1644.

4-[4-[1-[3,8-Dimethyl-5-(1-methylethyl)azulenyl]]-1,3-butadienyl]benzoic Acid (12). Phosphonate **24** (1.72 g, 6.0 mmol) was converted to its sodium salt by reaction with sodium hydride (0.24 g, 60% dispersion in mineral oil, 0.144 g, 6.0 mmol) in THF (30 mL). A solution of enal **14** (1.0 g, 3.97 mmol) in THF (20 mL) was added and the reaction mixture stirred at room temperature for 1 h. Conventional workup, column chromatographic purification using 25% ether–hexanes and recrystallization from ether–pentane gave butadienyl ester (0.5 g) as a red-brown solid in 32% yield: mp 123–126 °C dec; UV (ethanol) *448*, 642 nm; ^{1}H NMR δ 1.33 (6H, d, 7 Hz), 2.60 (3H, s, C3′-CH_3), 3.0 (1H, m), 3.01 (3H, s, C8′-CH_3), 3.91 (3H, s, OCH_3), 6.60 (1H, d, 15.4 Hz, C1-H), 6.85 (1H, dd, 10.8 and 15.4 Hz, C3-H), 6.85 (1H, d, 10.5 Hz, C7′-H), 7.16 (1H, dd, 10.9 and 15.4 Hz, C2-H), 7.23 (1H, dd, 1.9 and 10.7 Hz, C6′-H), 7.48 (2H, d, 8.4 Hz, phenyl H), 7.66 (1H, d, 14.9 Hz, C4-H), 7.88 (1H, s, C2′-H), 7.96 (1H, d, 2 Hz, C4′-H), 7.97 (2H, d, 8.3 Hz, phenyl H) ppm.

To the above ester (0.5 g, 1.3 mmol) dissolved in 50% THF–methanol (50 mL) was added a solution of KOH (2.5 g, 45 mmol) in 80% aqueous methanol (20 mL). After 0.5 h of gentle reflux the reaction mixture was worked up in the usual manner to afford pure acid **12** (0.45 g) as a dark solid in 94% yield: mp 242–244 °C; IR (film) 1674 (C=O) cm^{-1}; UV (ethanol) 288, 343, *442*, 650 nm; ^{1}H NMR (acetone-d_6) δ 1.28 (6H, d, 7 Hz), 2.54 (3H, s, C3′-CH_3), 2.8 (bs, CO_2H), 2.97 (3H, s, C8′-CH_3), 6.66 (1H, d, 15.5 Hz, C1-H), 6.89 (1H, d, 10.6 Hz, C7′-H), 6.93 (1H, dd, 10.8 and 15.0 Hz, C3-H), 7.29 (1H, dd, 1.0 and 10.3 Hz, C6′-H), 7.34 (1H, dd, 10.8 and 15.5 Hz, C2-H), 7.56 (2H, d, 8.2 Hz, phenyl H), 7.76 (1H, d, 15.0 Hz, C4-H), 7.93 (1H, s, C2′-H), 7.94 (2H, d, 8.0 Hz, phenyl H), 8.00 (1H, d, 1.7 Hz, C4′-H) ppm; exact mass (EI) calcd for $C_{28}H_{26}O_2$ (M^+) 370.1933, found 370.1921.

Methyl 4-[2-(1-Azulenyl)ethenyl]benzoate and 4-[2-(1-Azulenyl)ethenyl]benzoic Acid (13). Treatment of azulene-1-carboxaldehyde (1.05 g, 6.7 mmol) in THF (10 mL) with the lithio derivative of methyl 4-[(diethylphosphono)methyl]benzoate (**24**) (4.29 g, 15 mmol) in THF (25 mL) in the usual manner gave the requisite ester (0.41 g) as a dark solid in 21% yield: ^{1}H NMR δ 3.92 (3H, s, OCH3), 7.10–7.25 (3H, m), 7.42 (1H, d, 4.2 Hz), 7.57 (1H, t, 9.8 Hz), 7.62 (2H, d, 8.5 Hz, phenyl H), 7.83 (1H, d, 16.0 Hz, vinyl H), 8.02 (2H, d, 8.4 Hz, phenyl H), 8.2–8.3 (2H, m), 8.51 (1H, d, 9.8 Hz) ppm. Base hydrolysis of this ester (0.40 g, 1.4 mmol) with excess KOH (1.3 g, 23 mmol) in 80% aqueous methanol (25 mL) for 0.5 h at gentle reflux gave crude acid **13** which was column chromatographed using THF. Further purification by recrystallization from ethyl acetate–hexanes gave pure benzoic acid **13** (0.23 g) as a black solid in 60% yield: mp 265–268 °C dec; IR (film) 1672 cm^{-1}; UV (ethanol) 261, 318, 352, *406*, 630 nm; ^{1}H NMR δ 2.80 (bs, CO_2H), 7.21 (1H, t, 10.0 Hz), 7.25 (1H, t, 9.8 Hz), 7.38 (1H, d, 15.9 Hz, vinyl H), 7.47 (1H, d, 4.2 Hz), 7.66 (1H, t, 10.0 Hz), 7.80 (2H, d, 8.5 Hz, phenyl H), 8.01 (2H, d, 8.3 Hz, phenyl H), 8.11 (1H, d, 16.4 Hz, vinyl H), 8.34 (1H, d, 9.3 Hz), 8.39 (1H, d, 4.2 Hz), 8.78 (1H, d, 10.0 Hz) ppm; exact mass (EI) calcd for $C_{19}H_{14}O_2$ (M^+) 274.0994, found 274.0997.

Biological Testing: Cells and Cell Culture Conditions. The C3H10T1/2 cell line of murine embryo fibroblasts was used throughout this study.[43] Its response to carcinogens, to retinoids, and to many other modulators of carcinogenesis has been extensively studied. In general its behavior is highly predictive of in vivo responses to these agents.[44]

Assays for Inhibition of Neoplastic Transformation. Cells were treated with a transforming dose of 3-methylcholanthrene (3-MCA, 3 μg/mL) for 24 h and then, after an additional 7-day culture, with the stated retinoid dissolved in acetone (final concentration 0.5%). The retinoid was renewed after each weekly medium change. Cultures were maintained for 4 weeks after which they were fixed, stained, and scored for morphologically transformed foci. Details of this assay system and its use in testing cancer chemopreventive agents have been previously described.[30,45] As a negative control we used acetone; as positive control (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic Acid (TTNPB) obtained as a gift from Hoffmann-La Roche (Ro 13-7410) and previously shown to be highly potent in C3H10T1/2 cells.[28] We did not use retinoic acid as a positive control because it is rapidly metabolized by these cells.[46]

Assays for Gap Junctional Communication. Replicate cultures to those used for the transformation assays, but which had not been exposed to 3-MCA, were treated with retinoids as above and probed for gap junctional communication after 7, 14, and 21 days of treatment. In this assay representative cells were microinjected with the junctionally permeant fluorescent dye Lucifer Yellow (10% in 0.33 M LiCl) and the number of fluorescent adjacent cells counted after 10 min as previously described.[28]

Assays for Expression of Connexin 43. Cultures of C3H10T1/2 cells were treated with retinoids for 7 days beginning after cells had reached confluence. Cells were then harvested and lysed and total proteins subjected to polyacrylamide gel electrophoresis and immunoblotting (Western blotting) as described.[29] As primary antibody we used a rabbit polyclonal IgG raised against the predicted C-terminal amino acid residues (positions 368–382) of connexin 43. The position of bound IgG was detected by ^{125}I-labeled protein A. Details of this antibody and Western blotting protocols are described in ref 29.

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