# Conformationally Defined 6-*s*-*trans*-Retinoic Acid Analogs. 2. Selective Agonists for Nuclear Receptor Binding and Transcriptional Activity

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We recently demonstrated in animal models that a new conformationally defined RA isomer (Vaezi et al. *J. Med. Chem.* **1994**, *37*, 4499–4507) was as effective as RA in the prevention of skin papillomas but was less toxic. In order to provide more details concerning this improved action, we report here the preparation of a homologous conformationally defined 6-*s*-*trans*-retinoid (**1**) and investigate its ability to interact with proteins and to activate gene expression. Four configurational isomers of **1** were evaluated in binding assays for cellular retinoic acid binding protein, CRABP (isolated from chick skin); CRABP-I and CRABP-II (cloned from mouse); nuclear retinoic acid receptors (RARs); and nuclear retinoid X receptors (RXRs). In each assay the *all-E*-isomer of this retinoid had an activity that was comparable to that of (*all-E*)-RA. However, the 9*Z*-isomer was at least 200-fold less active than (*all-E*)-RA in binding to different RARs, while it was only 6–20 times less active than (9*Z*)-RA in binding to different RXRs. In an *in vivo* transient transfection assay, the *all-E*-isomer activated a reporter gene containing a retinoic acid response element (RARE) with efficiency similar to (*all-E*)-RA when expression vectors for either RARα, RAR$\beta$, RAR$\gamma$ alone or RARα together with RXRα were cotransfected. In contrast, the 9*Z*-isomer was much less active than (9*Z*)-RA in the same assay systems. However, (9*Z*)-**1** efficiently enhanced the DNA binding and transactivational activity of RXRα homodimers. Taken together, these studies demonstrate that the *all-E*- and 9*Z*-isomers of this retinoid are selective and potent agonists of RAR and RXR binding and activation.

Vitamin A acid, retinoic acid (RA), is essential for diverse biological events.[1,2] Since RA is capable of controlling gene expression, its use as a cancer chemopreventive or a chemotherapeutic agent has been extensively investigated using animal models.[3] Even though several studies have shown that RA may be effective in the prevention of cancer, its clinical use in therapy has been limited due to toxicity[4] and teratogenicity.[5]

The pleiotropic effects of this vitamin may be explained by a large family of nuclear receptors, which are related to the steroid/thyroid hormone superfamily of receptors and collectively act as ligand-dependent transcription factors for different genes.[6] In 1987, the first example of a nuclear retinoic acid receptor,[7] RARα, was discovered and shown to be activated by (*all-E*)-RA. Subsequently, two other subtypes, RAR$\beta$ and RAR$\gamma$, were identified with each subtype containing several different isoforms. Later, a second family of retinoid X receptors, RXRα, RXR$\beta$, or RXR$\gamma$, was isolated,[8] and only the 9*Z*-configuration of RA, rather than (*all-E*)-RA, was implicated in RXR binding[9] and RXR homodimer activation.[10] After this discovery, it was shown that in addition to (*all-E*)-RA, (9*Z*)-RA was capable of binding to RARs and activating both RAR homodimers and RAR/RXR heterodimers.[11]

Interestingly, both the therapeutic and toxicologic effects of RA have been suggested to be mediated by RARs, RXRs, and binding proteins.[12] Studies indicate that disorders in skin may be associated with abnormal expression of retinoic acid receptors.[13] In order to improve the therapeutic ratio of RA and to provide useful pharmacological probes for understanding the actions of the natural vitamin, new retinoid analogs that selectively activate individual nuclear receptors are needed. Starting in 1990, several groups[14] have reported the synthesis of RAR-selective retinoids and, more recently, others[15] have generated RXR-selective ligands. To date, all examples of nuclear receptor-selective retinoids contain substituted aromatic groups replacing the polyene chain of the natural vitamin.

For the natural vitamin, 6-*s*-*cis* and 6-*s*-*trans* conformers rapidly interconvert in solution[16] but only one conformer is selectively bound by proteins which recognize vitamin A.[17] We have designed two series of conformationally defined RA analogs.[18] By incorporating a dimethylene bridge, a 6-*s*-*cis* or 6-*s*-*trans* orientation of the polyene chain and terminal double bond is maintained without interconversion, while the rest of the polyene chain is identical to that of RA. Recently we reported[19] the synthesis of one 6-*s*-*trans* retinoid (UAB7) and showed that the biological activities of the

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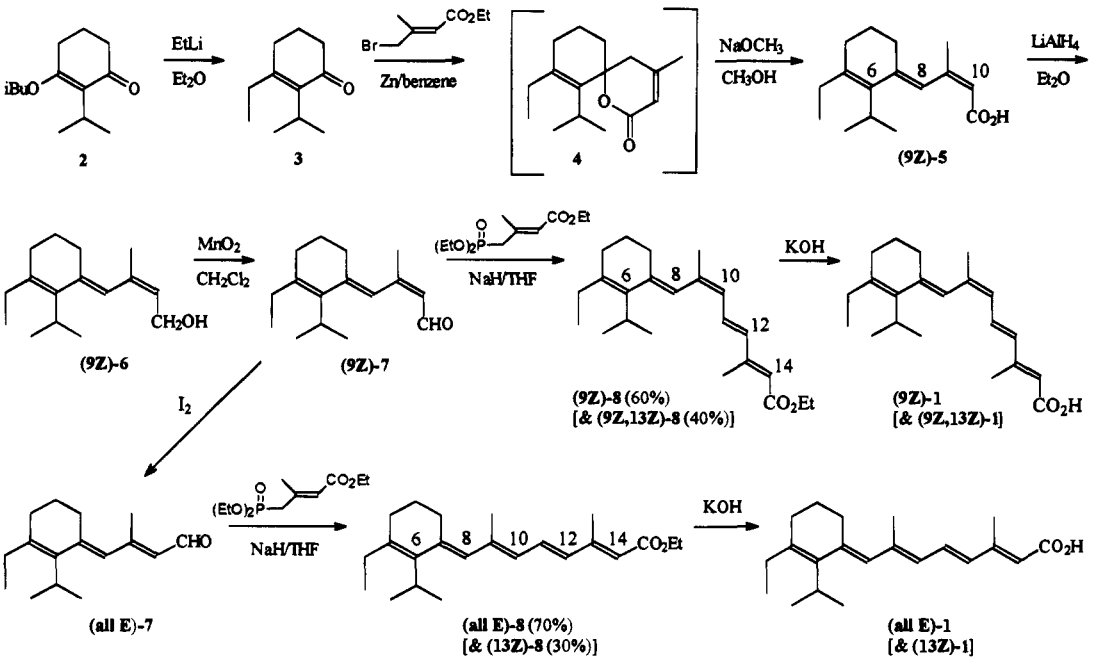
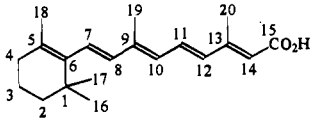
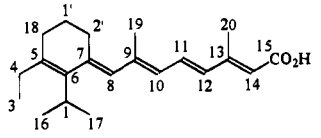
**Scheme 1**




*all-E*-isomer of this retinoid was as good as (*all-E*)-RA, but it had modestly reduced toxicity. Here we generate a more elaborated example (UAB8) of a 6-*s-trans* retinoid (retinoid **1**) and investigate the ability of four *E*/*Z*-isomers to bind to cellular retinoic acid binding proteins and nuclear receptors and to activate gene expression. We find that (*all-E*)-**1** selectively binds to RARs and activates RAR/RXR heterodimers. However, in contrast to (9*Z*)-RA, (9*Z*)-**1** selectively interacts with RXR nuclear receptors. These results reveal that conformations of single bonds in the C6–C9 region of the polyene chain are important for selectivity.



6-s-*trans* retinoic acid



retinoid 1

**Chemistry.** The synthesis of retinoid **1**, summarized in Scheme 1, was accomplished from 3-(isobutyloxy)-2-isopropyl-2-cyclohexenone (**2**) using essentially the same procedures that we previously reported[19] for the preparation of a homolog of **1**. Experimental yields and selected data for intermediates and products in Scheme 1 are summarized in Table 1.

The earlier synthesis[19] suffered from two low-yielding steps. The first involved the Reformatsky reaction of 2-isopropyl-3-methyl-2-cyclohexenone (e.g., as in the conversion of **3** to **4** in Scheme 1), which previously resulted in 30% recovered starting material and only 60% conversion (based on unrecovered starting material). Here we report an optimized Reformatsky procedure for these enones (e.g., **3**) that not only results in much higher yield (≥80%) without recovery of starting material but directly produces acid (9*Z*)-**5** without the isolation of intermediate lactone **4**. (Note that a vitamin A numbering scheme is being used for retinoid **1** and precursors.) Unlike previous reports by Robinson et al.[20] and Vaezi et al.,[19] lactone **4** was not detected and was assumed to be an intermediate in this process.

The second problem with the earlier methodology involved the $MnO_2$ oxidation of a homolog of alcohol (9*Z*)-**6** to form the aldehyde, which was similarly troubled by recovery of starting material and relatively poor percent conversion. In our earlier report,[19] we described an improved procedure involving the inclusion of powdered molecular sieves, which we believe mechanically cleans the $MnO_2$ surface. Here we report a modified procedure utilizing sea sand and $MnO_2$ which gives even better results.

**Table 1.** Selected Data for New Compounds Produced in Scheme 1

| compd no. | % yield | $R_f$[a] | UV/vis[b] $\lambda_{max}$ | UV/vis[b] $\epsilon_{max}$ | IR[c] C=O | IR[c] C=C | GC/MS *m/z* |
|---|---|---|---|---|---|---|---|
| **3** | 87 | 0.56 | 235 | 13700 | 1665 | 1611 | 166 |
| (9*Z*)-**5** | 81 | 0.39 | 323 | 10500 | 1690 | 1618 | 248 |
| (9*Z*)-**6** | 96 | 0.26 | 253 | 13700 | —[d] | 1653 | 234 |
| (*all-E*)-**7** | 52[e] | 0.22 | 326 | 10100 | 1661 | 1574 | 232 |
| (9*Z*)-**7** | 86[e] | 0.36 | 300 | 6700 | 1680 | 1611 | 232 |
| (*all-E*)-**8** | 85 | 0.67 | 365 | ND | 1711 | 1605 | 342[f] |
| (9*Z*)-**8** | 90 | 0.69 | 330 | ND | 1711 | 1603 | 342[f] |
| (13*Z*)-**8** | 85 | 0.73 | 369 | ND | 1711 | 1605 | 342[f] |
| (9*Z*,13*Z*)-**8** | 90 | 0.71 | 332 | ND | 1711 | 1603 | 342[f] |
| (*all-E*)-**1** | 95 | 0.25 | 376 | 29700 | 1680 | 1595 | 314[f] |
| (9*Z*)-**1** | 96 | 0.24 | 336 | 22100 | 1680 | 1595 | 314[f] |
| (13*Z*)-**1** | 94 | 0.34 | 375 | 26600 | 1676 | 1593 | 314[f] |
| (9*Z*,13*Z*)-**1** | 92 | 0.28 | 340 | 19500 | 1675 | 1590 | 314[f] |

[a] Values obtained on silica gel using diethyl ether in hexane as an eluent: 10% $Et_2O$, **7**; 20% $Et_2O$, **1**, **3**, and **8**; 30% $Et_2O$, **5** and **6**. [b] The wavelength maximum (nm) and extinction coefficients ($M^{-1}$ $cm^{-1}$) were obtained in cyclohexane at room temperature. ND means not determined. [c] The IR stretching frequencies ($cm^{-1}$) were obtained on KBr films. [d] The hydroxyl group stretching frequency was observed at 3322 $cm^{-1}$. [e] Based on unrecovered starting material. [f] The electron impact spectra were taken using the direct insertion probe.

**Table 2.** HPLC Retention Times of Four *E/Z*-Isomers of Retinoid **8** and Retinoid **1**

| peak | retinoid *E/Z*-isomer | retinoid **8** normal-phase retention times[a] (min) | retinoid **1** reverse-phase retention times[b] (min) | retinoid **1** isomeric purity[c] (%) |
|---|---|---|---|---|
| A | 13*Z* | 64.0 | 18.1 | >98 |
| B | 9*Z*,13*Z* | 70.8 | 19.5 | >98 |
| C | 9*Z* | 72.8 | 20.7 | >90[d] |
| D | *all-E* | 89.6 | 22.9 | >97 |

[a] The separation of retinoid **8** isomers was performed on a Whatman Partisil 10 M20/50 column (500 × 22 mm i.d.) using 1% $Et_2O$, 0.5% THF in hexane with a flow rate of 5 mL/min. Peaks were monitored at 340 nm by UV/vis detection. [b] The separation of retinoid 1 isomers was performed on a Chromanetics Spherisorb ODS column (250 × 4.6 mm i.d.) using 1% acetic acid in acetonitrile (3:7) with a flow rate of 1.0 mL/min. Peaks were monitored at 340 nm by UV/vis detection. [c] The isomeric purity of retinoid 1 isomers was determined from reverse-phase peak areas which were corrected for small differences in extinction coefficients at 340 nm (see Figure 1). The total area was determined from the sum of each corrected isomer peak area. The isomeric purity was calculated as the corrected area/total area X 100. [d] The major contaminant was the 9*Z*,13*Z*-isomer (10%).

The $I_2$-catalyzed isomerization of (9*Z*)-**5** yielded a 1:2 ratio of *all-E*- and 9*Z*-isomers. The *E/Z*-configuration was confirmed by NOE studies. For (9*Z*)-**7**, a large NOE (23%) was observed to the H-10 vinyl proton and no NOE to H-11 when the 9-methyl protons were irradiated. This is consistent with a 9*Z*-configuration. Irradiation of the isopropyl methyl groups resulted in large NOE (28%) to the H-8 vinyl proton, which is consistent with a 7*E*-configuration. The *all-E*-configuration of **7** was confirmed by a large NOE (27%) from the isopropyl methyl groups to the H-8 vinyl proton and a large NOE (30%) between 9-methyl and H-11.

Finally, the Horner–Emmons condensations of either the *all-E*- or 9*Z*-isomers of aldehyde **7** (Scheme 1) produced esters **8** as mixtures of *E,Z*-configurations using procedures exactly as described previously for a related analog.[19] Four main isomers (*all-E*; 9*Z*; 13*Z*; and 9*Z*,13*Z*) were preparatively separated by HPLC on silica (Table 2), using methods similar to those we previously described for a retinoid **8** homolog[19] or for other retinoid esters.[21] The *all-E*- and 13*Z*-isomers (peak A at 64.0 min and peak D at 89.6 min) were separated cleanly from the two other isomers of intermediate retention times. The 9*Z*- and 9*Z*,13*Z*-isomers were pooled and separated by HPLC on silica using a less polar solvent (0.5% $Et_2O$ in hexane).

The $^1H$ NMR chemical shift assignments of *E/Z*-isomers of ester **8** (Table 3) were made by selective decoupling experiments and COSY 2D experiments. $^1H$ NMR chemical shifts of H-8–H-14 were comparable to similar retinoids.[19] Both these shifts and the $^{13}C$ NMR shifts are very sensitive to different *E/Z*-configurations as reported by Vaezi et al.[22] and by Hope et al.[23] Individual *E/Z*-isomers of **8** were hydrolyzed to the corresponding acid in KOH.[24] Using NMR spectroscopy, the hydrolysis was shown to proceed without *E/Z*-isomerization. The extinction coefficients were determined for each isomer by a Beer's law titration. The UV/vis spectrum of each isomer (Figure 1) showed that the wavelength maxima of (*all-E*)-**1** and (13*Z*)-**1** were near 365 nm, while the maxima for the 9*Z*- and 9*Z*,13*Z*-isomers were blue-shifted to 335–340 nm. The wavelength maxima of both (*all-E*)- and (13*Z*)-**1** were red-shifted by about 16 nm compared to the spectra of RA isomers. Using methods developed for the detection of RA isomers,[25] the isomeric purity of each *E/Z*-isomer of acid **1** was determined by reverse-phase HPLC on a C18 column using UV detection at 340 nm (Table 2). The purities were typically greater than 95% for each isomer; the 9*Z*-isomer was less pure and contained 10% of the 9*Z*,13*Z*-isomer.

**Biology.** In order to study the ability of the four stereoisomers of retinoid **1** to recognize RA-binding sites within protein receptors, the concentrations causing 50% inhibition ($IC_{50}$) and the apparent dissociation constants ($K_d'$) were determined for CRABPs, RARs, and RXRs. The four isomers were initially evaluated in a protein binding assay developed by Sani and co-workers.[26] Each isomer was tested for its ability to inhibit the binding of (*all-E*)-RA to CRABP isolated from chick skin. Next, the $K_d'$ values were determined for each isomer complexed with mouse *apo*-CRABP-I or *apo*-CRABP-II using a fluorometric titration of ligand and *apo*-protein according to the methods developed by Li and co-workers.[27] Using similar methods, the binding affinities of these isomers were also determined to the ligand-binding (DEF) domain of human RXRα, which was cloned according to the methods of Cheng et al.[28] The $IC_{50}$ values for each *E/Z*-isomer of retinoid **1** were next determined in an assay for the inhibition of the binding of (*all-E*)-RA to RARα, RARβ, and RARγ (cloned from mouse) using methods described by Levin and co-workers.[11] Similar studies were also performed for the inhibition of the binding of (9*Z*)-RA to RXRα, RXRβ, and RXRγ (mouse clone).[29] Finally, each isomer was evaluated for the efficiency of activating RARα, -β, or -γ homodimers, RXRα homodimers or RXRα/RARα heterodimers using a CAT reporter gene containing the TREpal according to a method described by Zhang et al.[30]

## Results and Discussion

Retinoid **1** contains ethyl and isopropyl substituents on the cyclohexenyl ring. When the polyene chain and carboxylic acid groups of (*all-E*)-RA and (*all-E*)-**1** are overlaid, the ethyl group substituent at C-5 on retinoid **1** can occupy space similar to the C-3 and C-4 methylene groups in the trimethylcyclohexenyl ring of RA. Likewise, the isopropyl group of **1** can occupy space similar to C-1 and the C-1 *gem*-dimethyl groups (C-16 and C-17) of RA. Except for C-2 of RA, the substituted cyclohexenyl ring of (*all-E*)-**1** mimics the majority of the trimethylcyclohexenyl ring of RA and maintains a 6-*s-trans*-geometry of the polyene chain. Additionally, the C-1′ and C-2′ methylene carbons of retinoid **1** add extra steric volume near the C-9 methyl group (C-19) that is not in common with RA. A model of a similar retinoid overlaid with RA was presented by Vaezi et al.[19]

Four isomers of retinoid **1** were first evaluated in the CRABP-binding assay developed by Sani and co-workers.[26] The $IC_{50}$ values are given in Table 4 and compared to those of (*all-E*)-RA and (13*Z*)-RA. As shown, (*all-E*)-**1** was nearly as effective as (*all-E*)-RA in competing for the single ligand binding site in CRABP isolated from chick skin. These results are consistent with those previously determined by Vaezi et al.[19] for a related 6-*s-trans*-retinoid. The $IC_{50}$ value for (13*Z*)-**1** was nearly identical to that of (*all-E*)-**1** (Table 4). Previous studies by Sani[26b] using (13*Z*)-RA in this assay

**Table 3.** $^1$H NMR Chemical Shifts (300 MHz) of Retinoids **1**–**8** in $CDCl_3$ (ppm, TMS)[a]

| isomer | H-1′ | H-2′ | H-1 | H-3 | H-4 | H-8 | H-10 | H-11 | H-12 | H-14 | H-16 | H-18 | H-19 | H-20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **3** | 1.86 | 2.30 | 2.87 | 1.06 | 2.23 | | | | | | 1.16 | 2.30 | | |
| (9*Z*)-**5** | 1.61 | 2.28 | 3.08 | 1.00 | 2.11 | 5.68 | 6.63 | | | | 1.23 | 2.13 | 12.05 | |
| (9*Z*)-**6** | 1.59 | 2.11 | 3.06 | 0.99 | 2.11 | 5.85 | 5.45 | 4.01 | | | 1.22 | 2.11 | 1.78 | |
| (9*Z*)-**7** | 1.58 | 2.16 | 3.07 | 0.98 | 2.11 | 6.03 | 5.89 | 9.52 | | | 1.19 | 2.11 | 1.98 | |
| (*all-E*)-**7** | 1.62 | 2.48 | 3.10 | 1.03 | 2.17 | 6.05 | 5.94 | 10.03 | | | 1.23 | 2.15 | 2.28 | |
| (*all-E*)-**8** | 1.61 | 2.47 | 3.09 | 1.02 | 2.15 | 6.05 | 6.07 | 6.92 | 6.23 | 5.75 | 1.22 | 2.12 | 1.99 | 2.35 |
| (9*Z*)-**8** | 1.60 | 2.09 | 3.12 | 1.02 | 2.15 | 5.96 | 6.01 | 6.65 | 6.17 | 5.73 | 1.25 | 2.13 | 1.90 | 2.25 |
| (13*Z*)-**8** | 1.59 | 2.47 | 3.08 | 1.01 | 2.16 | 6.04 | 6.19 | 6.91 | 7.73 | 5.61 | 1.22 | 2.12 | 1.98 | 2.06 |
| (9*Z*,13*Z*)-**8** | 1.59 | 2.09 | 3.11 | 1.02 | 2.15 | 5.96 | 6.11 | 6.64 | 7.65 | 5.59 | 1.25 | 2.10 | 1.90 | 1.95 |
| (*all-E*)-**1** | 1.61 | 2.46 | 3.08 | 1.01 | 2.11 | 6.04 | 6.09 | 6.99 | 6.24 | 5.77 | 1.22 | 2.13 | 1.98 | 2.32 |
| (9*Z*)-**1** | 1.61 | 2.11 | 3.12 | 1.03 | 2.16 | 5.96 | 6.01 | 6.70 | 6.20 | 5.75 | 1.26 | 2.13 | 1.91 | 2.25 |
| (13*Z*)-**1** | 1.61 | 2.49 | 3.08 | 1.01 | 2.14 | 6.06 | 6.20 | 6.96 | 7.69 | 5.64 | 1.22 | 2.16 | 1.99 | 2.10 |
| (9*Z*,13*Z*)-**1** | 1.60 | 2.11 | 3.12 | 1.02 | 2.13 | 5.97 | 6.14 | 6.70 | 7.63 | 5.62 | 1.25 | 2.15 | 1.92 | 1.99 |

[a] Retinoids were numbered analogously to retinoic acid for easy comparison.

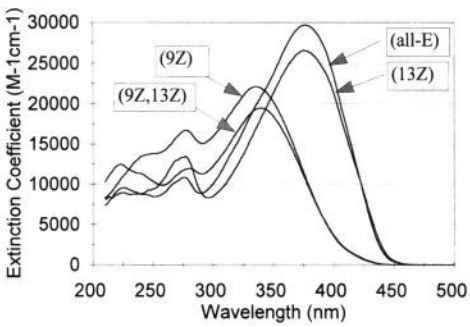



**Figure 1.** UV/vis spectrum of four *E*/*Z*-isomers of retinoid **1** measured in cyclohexane at room temperature.

**Table 4.** Inhibition Concentrations at 50% and Apparent Dissociation Constants for Binding of Retinoid **1** and Retinoic Acid Isomers to Mouse and Chick Skin CRABPs and hRXRα (DEF) Domain

| retinoid isomer | cCRABP $IC_{50}$ (μM)[a] | mCRABP-1 $K_d$ (nM) [stoichiometry] | mCRABP-II $K_d$ (nM) [stoichiometry] | hRXRα (DEF) $K_d$ (nM) [stoichiometry] |
|---|---|---|---|---|
| (*all-E*)-RA | 0.6 | 0.4 ± 0.3[b] [1.2] | 2 ± 1[b] [1.0] | >200[c] |
| (13*Z*)-RA | 0.9[d] | >200[b] | >200[b] | NR[c] |
| (9*Z*)-RA | >500 | 210 ± 150[b] | 190 ± 120[b] | 3 ± 0.5[c] [1.1] |
| (*all-E*)-**1** | 0.7 | 0.3 ± 0.02 [0.9] | 1.4 ± 0.2 [1.2] | >200 |
| (13*Z*)-**1** | 1.0 | >200 | >200 | >200 |
| (9*Z*)-**1** | >500 | >200 | >200 | 16 ± 0.6 [1.1] |
| (9*Z*,13*Z*)-**1** | >500 | >200 | >200 | >200 |

[a] The $IC_{50}$ values were determined by a probit analysis. The standard deviation was less than ±0.1 μM for the (*all-E*)- and (13*Z*)-isomers. [b] Values reported by Norris et al.[27] Binding stoichiometry was not reported for some isomers due to the $K_d$ values approaching the water solubility of RA. [c] Values reported by Cheng et al.[28] NR means not reported. [d] Value reported in Vaezi et al.[19]

showed that this isomer competes nearly as well as (*all-E*)-RA for the binding site in CRABP (chick skin) (Table 4). The other stereoisomers were weak binders, and the $IC_{50}$ values were not determined.

The apparent dissociation constants for the complex of each isomer with mCRABPs and with hRXR-α (DEF) were next determined by the procedures developed by Li and co-workers.[27] Ligand binding to *apo*-protein was monitored by using the fluorescence quenching of the aromatic residues upon ligand addition as described by Cogan et al.[31] Fluorometric titrations of retinoid **1** isomers were performed with several recombinant proteins: *apo*-mCRABP-I, *apo*-mCRABP-II, or *apo*-hRXRα (DEF). Initially the titrations used micromolar protein concentrations to survey binding of each ligand to the CRABPs and hRXRα (DEF). These titrations showed that only the *all-E*-isomer bound tightly to mCRABP-I and mCRABP-II. Likewise the 9*Z*-isomer bound tightly only to hRXRα (DEF). These titrations established an 1:1 stoichiometry of the ligand–protein complex (Table 4). The other isomers, including (13*Z*)-**1**, were poor binders to these proteins and receptor domains ($K_d'$ > 200 nM). The apparent dissociation constants for the isomers which bind weakly to the retinoid-binding proteins/domains cannot be accurately determined due to the poor solubility of retinoids in aqueous solutions.[32] The difference in affinities and $IC_{50}$ values of (13*Z*)-**1** for mouse and chick skin CRABPs (Table 4) is most reasonably attributed to subtle differences in the RA-binding site of these two proteins from different sources.

Fluorometric titrations of the tight-binding retinoid isomers were performed at lower total protein concentrations (10–25 nM) to more accurately determine the apparent dissociation constants (Table 4).[33] The dissociation constants for the protein–ligand complex with (*all-E*)-**1** was 0.3 nM (mCRABP-I) and 1.4 nM (mCRABP-II). Previously, Li and co-workers[27] determined the binding affinities of (*all-E*)-RA to these CRABPs using the same methods; the dissociation constants obtained for (*all-E*)-RA (see Table 4) were indistinguishable from those of (*all-E*)-**1**. Fluorometric titrations of (9*Z*)-**1** were performed with hRXRα (DEF) using a 20 nM total protein concentration. The apparent dissociation constant was determined to be 16 nM (Table 4). Even though the affinity of this isomer to hRXR-α (DEF) was very high, the dissociation value was about 5-fold greater than that observed by Cheng et al.[28] for (9*Z*)-RA, indicating that this isomer is a weaker binder than the natural ligand.

To further probe the binding interaction of these retinoid isomers with receptors, each isomer was evaluated for its ability to inhibit the binding of [$^3$H]-(*all-E*)-RA and [$^3$H]-(9*Z*)-RA to the RAR and RXR subtypes, respectively. To survey the inhibition process, RARα was exposed to 1000 nM of each of the four isomers and 5 nM of [$^3$H]-(*all-E*)-RA. This was compared to a positive control using 1000 nM of unlabeled (*all-E*)-RA and a background correction using no retinoid. Only the (*all-E*)-**1** displaced the radioactive ligand as well as (*all-E*)-RA (see Table 5 for details).

The $IC_{50}$ values with RARα, RARβ, and RARγ were determined for the three isomers of **1** which were effective inhibitors. The $IC_{50}$ values for (*all-E*)-**1** were between 6 and 11 nM for the different RARs (Table 5).

**Table 5.** Inhibition Concentrations at 50% (nM) for the Binding of Retinoid **1** and Retinoic Acid Isomers to Mouse Retinoic Acid Receptors (mRARs) and Retinoid X Receptors (mRXRs)

| retinoid isomer | $IC_{50}$ (nM)[a] | | | $IC_{50}$ (nM)[a] | | |
|---|---|---|---|---|---|---|
| | mRARα | mRARβ | mRARγ | mRXRα | mRXRβ | mRXRγ |
| (*all-E*)-RA | 5[b] | 5[b] | 4[b] | >50000[c] | >50000[c] | >50000[c] |
| (13*Z*)-RA | >1000[d] | >1000[d] | >1000[d] | >50000[c] | >50000[c] | >50000[c] |
| (9*Z*)-RA | 31[b] | 8[b] | 60[b] | 73[c] | 117[c] | 85[c] |
| (*all-E*)-**1** | 11[e] | 6 | 10 | >1000[f] | NT | NT |
| (13*Z*)-**1** | 900[e] | 371 | 708 | >1000[f] | NT | NT |
| (9*Z*)-**1** | 1050[e] | 1130 | 1590 | 1500[f] | 640 | 1080 |
| (9*Z*,13*Z*)-**1** | >1000[e] | NT | NT | >1000[f] | NT | NT |

[a] The $IC_{50}$ values were determined by the methods of Allenby et al.[11,29] For $IC_{50}$ values with RARs, competition of unlabelled test retinoid was determined in the presence of 5 nM of [$^3$H]-(*all-E*)-retinoic acid. For RXRs, competition of unlabeled test retinoid was determined in the presence of 20 nM of [$^3$H]-(9*Z*)-retinoic acid. NT means not tested. The standard deviation was less than 10% of the $IC_{50}$ value. [b] Values reported by Allenby et al.[11] Based on repeted measurements, the estimated error is 20% of the mean. [c] Values reported by Allenby et al.[29] Based on repeated measurements, the estimated error is 20% of the mean. [d] Unpublished data by Levin and co-workers. [e] Using a single concentration of test retinoid (1000 nM) and [$^3$H]-(*all-E*)-RA (5 nM), the percent inhibition was 98% for (*all-E*)-**1**, 44% for (13*Z*)-**1**, 42% for (9*Z*)-**1**, and 11% for (9*Z*,13*Z*)-1. [f] Using a single concentration of test retinoid (1000 nM) and [$^3$H]-(9*Z*)-RA (20 nM), the percent inhibition was 0% for (*all-E*)-**1**, 0% for (13*Z*)-**1**, 50% for (9*Z*)-**1**, and 8% for (9*Z*,13*Z*)-**1**.

These values are comparable to those previously reported for (*all-E*)-RA by Allenby et al.[11] This indicates that the structural modifications for maintaining the 6-*s-trans* conformation in **1** do not interfere with the binding of the *all-E*-isomer to the RARs, consistent with the binding of this isomer to CRABPs from different sources (see Table 4). In contrast, the $IC_{50}$ values of (9*Z*)-**1** were 25−150 times larger than the values for (9*Z*)-RA. Apparently the structural modifications for this isomer are not well tolerated by the RARs. Either they prevent (9*Z*)-**1** from occupying a similar conformation in the binding pocket of the RARs or they introduce steric problems which interfere with proper binding. In contrast to (13*Z*)-RA, (13*Z*)-**1** binds to RARs with an affinity that is comparable to that of the 9*Z*-isomer (Table 5). The isomeric purity of (13*Z*)-**1** was greater than 98% (Table 3), and no *all-E*-isomer was present.

The four isomers of **1** were further evaluated for their ability to inhibit the binding of [$^3$H]-(9*Z*)-RA to RXRα. A preliminary screen was performed using a single concentration of test retinoid (1000 nM) and 20 nM of $^3$H-labeled (9*Z*)-RA. A positive control of 1000 nM (9*Z*)-RA efficiently inhibited the binding of labeled (9*Z*)-RA. Among the isomers of **1**, only (9*Z*)-**1** appreciably inhibited the binding of labeled (9*Z*)-RA. However, the percent inhibition of binding for (9*Z*)-**1** at 50-fold molar excess of ligand was considerably less than that observed for (9*Z*)-RA (Table 5). The inhibition observed for the other *E*/*Z*-isomers at 50-fold excess was less than 10%, and consequently, they were not studied further. The $IC_{50}$ values of (9*Z*)-**1** were also determined for RXRα, RXRβ, and RXRγ (Table 5). These values were between 5- and 20-fold larger than the corresponding values for (9*Z*)-RA reported by Allenby et al.[29] using the same methods (Table 5) and to our observed values. These data are also consistent with the $K_d'$ values reported in Table 4 using fluorometric titrations of the ligand and *apo*-hRXRα (DEF) domain.

To determine if the isomers of retinoid **1** exhibited functional activity within the cell, their ability to induce RAR- and RXR-mediated transcriptional activity was examined. The activities of these isomers were compared with those of (*all-E*)-RA and (9*Z*)-RA by transient transfection analysis of a reporter gene expressed in CV-1 cells according to methods developed by Graupner et al.[34] (Figure 2). Different subtypes of the RAR receptors (RARα, RARβ, and RARγ) were cotransfected with the $(TREpal)_2$-tk-CAT reporter gene into CV-1 cells. For each receptor subtype, (*all-E*)-**1** was equivalent to or better than (*all-E*)-RA in inducing the recep-

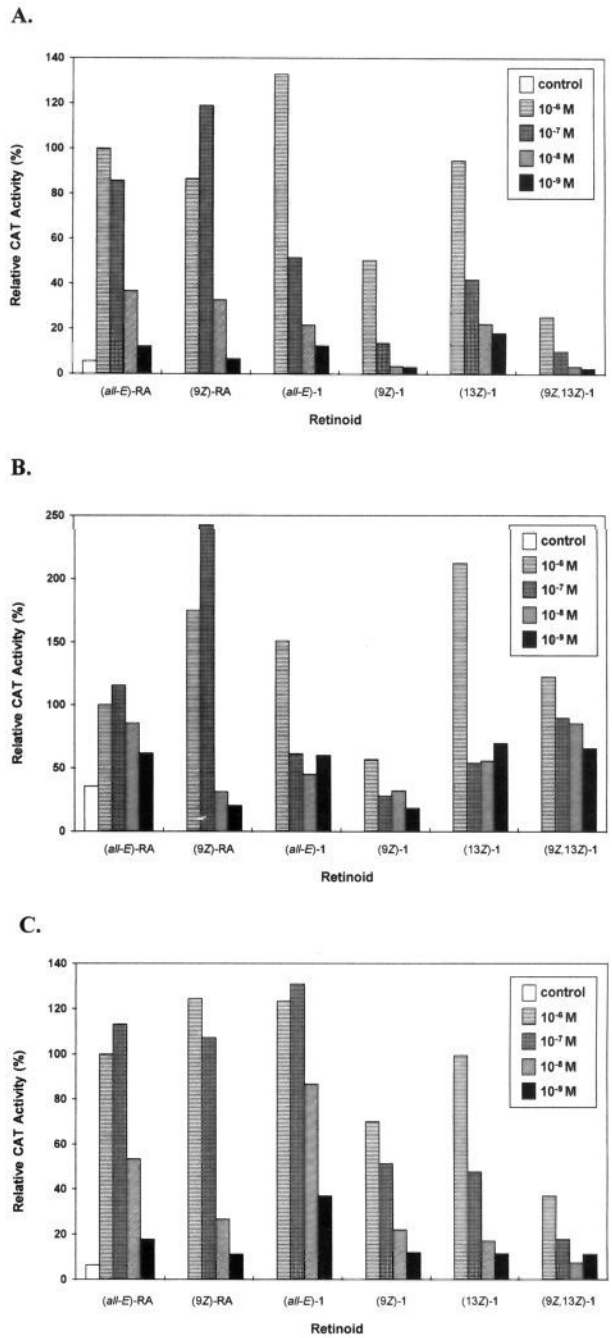



**Figure 2.** Analysis of RAR receptor-dependent transcriptional activation by (*all-E*)-RA and (9*Z*)-RA and four isomers of retinoid **1**: (*all-E*)-**1**, (9*Z*)-**1**, (13*Z*)-**1**, and (9*Z*,13*Z*)-**1** at $10^{-9}$–$10^{-6}$ M. (A) RARα-receptor transcriptional activation. (B) RARβ-receptor transcriptional activation. (C) RARγ-receptor transcriptional activation. The relative CAT activity is expressed as a percentage of activity relative to that induced by (*all-E*)-RA.

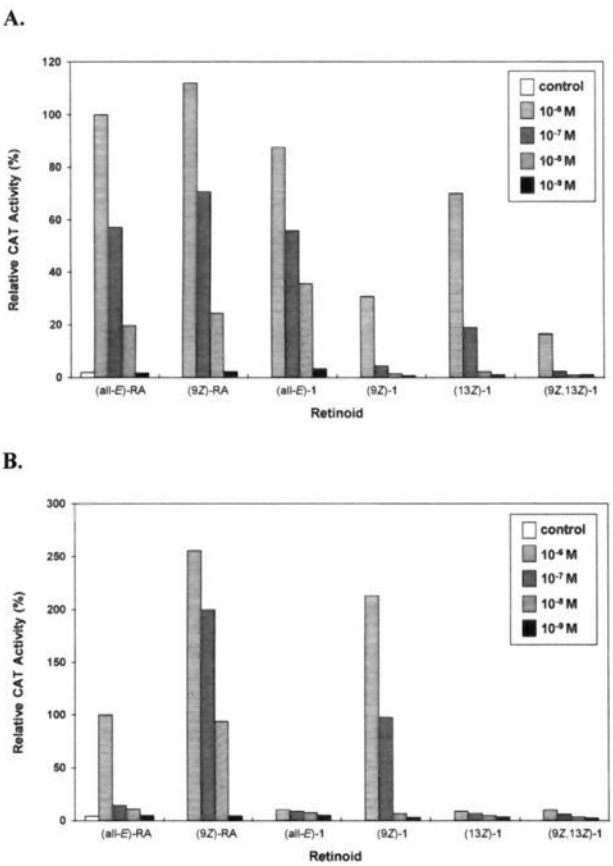



**Figure 3.** Analysis of RXR receptor-dependent transcriptional activation by (*all-E*)-RA and (9*Z*)-RA and four isomers of retinoid **1**: (*all-E*)-**1**, (9*Z*)-**1**, (13*Z*)-**1**, and (9*Z*,13*Z*)-**1** at $10^{-9}$–$10^{-6}$ M. (A) RXRα/RARα heterodimer–receptor transcriptional activation. (B) RXRα homodimer–receptor transcriptional activation.

tor-activated transcription of these genes (Figure 2). These results are consistent with the $IC_{50}$ values found for the binding of this isomer to the RARs (Table 5). Activation of RARs by (9*Z*)-RA was as good as (*all-E*)-RA (Figure 2). This is consistent with previous studies by Allenby et al.,[11] reporting that (9*Z*)-RA had equal or better activity than (*all-E*)-RA in inducing transcriptional activation in GAL4-RAR chimeric constructs. In contrast, (9*Z*)-**1** was much less efficient than either (9*Z*)-RA, (*all-E*)-RA, or (*all-E*)-**1** in the activation of gene transcription mediated by RAR subtypes. Consistent with their relative RAR binding profiles (Table 5), the activity of (13*Z*)-**1** was comparable to (9*Z*)-**1**, while (9*Z*,13*Z*)-**1** was the least active in these assays (Figure 2).

To evaluate the induction of RARα/RXRα heterodimer or RXRα homodimer activity by the test compounds, the $(TREpal)_2$-tk-CAT reporter gene was again used.[34] This reporter gene is activated by either RARα/RXRα heterodimers or RXRα homodimers as reported by Zhang et al.[10,30] in a transient transfection assay in CV-1 cells. When the effect on RARα/RXRα heterodimer activity of these compounds were studied (Figure 3A), (*all-E*)-**1** was as efficient as (*all-E*)-RA in activating the heterodimer gene reporter system. However, induction of the reporter gene by (9*Z*)-**1** was only apparent at $10^{-6}$ M, which was at least 100-fold less active than (9*Z*)-RA. When the effect on RXRα homodimer activity was studied, (*all-E*)-RA did not efficiently activate the

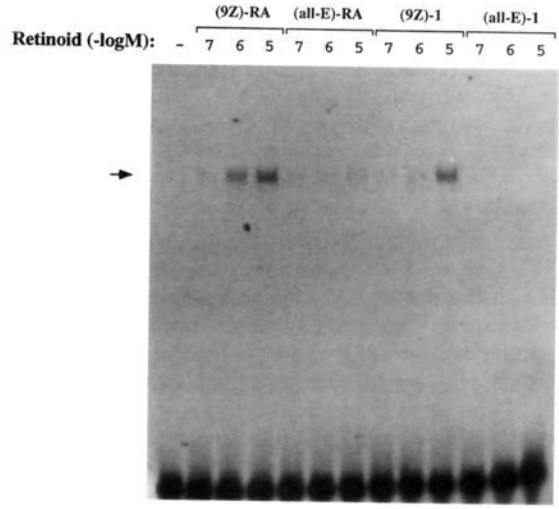



**Figure 4.** Gel shift experiments of RXR homodimer–DNA binding using a TREpal probe induced by different concentrations of RA and retinoid **1**. Arrow indicates the binding of RXRα homodimer complex.

reporter (Figure 3B). In contrast, (9*Z*)-RA efficiently activated this reporter gene which is consistent with previous observations by Zhang et al.[10] In contrast to its effects on RAR-mediated activation, (9*Z*)-**1**, like (9*Z*)-RA, caused significant activation at concentrations as low as $10^{-7}$ M. The activation by (9*Z*)-**1** was about 10-fold less potent than that observed for (9*Z*)-RA. This is consistent with the larger $K_d'$ (Table 4) and $IC_{50}$ (Table 5) values reported for the binding of this isomer to RXRα. While (*all-E*)-RA is a poor activator of this reporter gene system, (*all-E*)-**1** did not activate this homodimer concentrations up to $10^{-6}$ M (Figure 3). Likewise, the 13*Z*- or 9*Z*,13*Z*-isomers were both inefficient activators of this reporter gene. These results demonstrate that (9*Z*)-**1**, unlike (9*Z*)-RA, is highly selective for the activation of RXRα homodimers.

Interestingly, (13*Z*)-**1** had moderate activity in the RARα/RXRα heterodimer assay (Figure 3A). This isomer was not active in the RXRα homodimer assay (Figure 3B), nor did it bind effectively to RXRα (Tables 4 and 5). However, it partially activated RARα in the homodimer assay (Figure 2), consistent with its binding affinity to these receptors (Table 5). Assuming that isomerization to the *all-E*-isomer is not occurring *in vivo*, (13*Z*)-**1** most probably acts, like the *all-E*-isomers, through RARα interaction.

Previously, Zhang et al.[10] demonstrated activation of RXR homodimer activity by (9*Z*)-RA through the induction of RXR homodimer formation. To determine whether (9*Z*)-**1** could also induce RXR homodimer formation, a gel retardation experiment was carried out using TREpal as a probe. As shown in Figure 4, when *in vitro* synthesized RXR protein was mixed with (9*Z*)-RA, significant induction of RXR homodimer DNA binding was observed at concentrations as low as $10^{-7}$ M. When (9*Z*)-**1** was examined in the same manner, a significant RXR homodimer DNA binding was observed at $10^{-6}$ M (Figure 4). This approximately 10-fold reduction in potency for (9*Z*)-**1** as compared to (9*Z*)-RA is consistent with the differences observed in the transcriptional activation assay (Figure 3B) and RXR-binding assay (Table 5). Due to the probable partial conversion of (*all-E*)-RA to (9*Z*)-RA, (*all-E*)-RA slightly

enhanced the binding of RXR homodimers at high concentrations ($10^{-6}$ M). However, (*all-E*)-**1** had no effect on RXR homodimer binding at this concentration, consistent with the results obtained in the transcriptional assay (Figure 3B) and RXR-binding assays (Table 5).

In conclusion, we synthesized a second example (UAB8) of a new class of conformationally constrained 6-*s-trans*-retinoids (**1**). Previously, we showed that UAB7, a related *all-E*-retinoid, was as active as (*all-E*)-RA in the prevention of mouse skin papillomas but was less toxic. Since the synthetic approach employed for this class of conformationally defined 6-*s-trans*-retinoids allowed for the straightforward addition of other R groups to the cyclohexenyl ring, we prepared a more elaborated analog (**1**) that more fully mimics the steric volume of RA. We show in this study that the structural modification made in this series of retinoids generates *all-E*- and 9*Z*-isomers which are selective agonists of RAR and RXR binding and transcriptional activation. If the cancer chemopreventive and toxicity effects of retinoids are mediated through nuclear receptors, the similar activity and lower toxicity of this class of retinoids (as compared to RA) may result from the selective actions of these retinoids.

## Experimental Section

**Chemistry. General Methods.** $^1$H NMR spectra were obtained at 60 MHz on a Varian EM-360 spectrometer, a 300.1 MHz GE spectrometer (NT300) equipped with a 1180e Nicolet computer and 293c pulse programmer, or a 300.1 MHz Bruker ARX spectrometer equipped with a 5-mm $^1$H/$^{19}$F/$^{31}$P/$^{13}$C probe. NMR spectra were referenced internally to TMS. Steady-state NOE experiments were utilized to verify configurational assignments and were performed on degassed samples in sealed tubes as we previously described.[19] UV/vis spectra were recorded on a Perkin-Elmer Lambda 6 or AVIV 14DS spectrophotometer in cyclohexane solution (Fisher, Spectrograde). IR spectra were recorded using a Beckman AccuLab-4 or Nicolet FT IR spectrometer. Electron-impact mass spectra (70 eV) were obtained on a Hewlett-Packard 5985 GC/MS with an ultraperformance fused silica column. HPLC separations were performed on a Gilson HPLC gradient system using 25-mL pump heads and an ISCO V$^4$ variable wavelength detector. The column employed was a Whatman Partisil 10 M20/50 (500 × 22 mm i.d.). Preparative separations on silica were obtained with 5 mL/min flow rates using the indicated solvent systems. Reverse-phase HPLC separations on carboxylic acids were performed using a Beckman Model Gold Chromatography system interfaced to a personal computer and a Chromanetics Spherisorb ODS 5-$\mu$m column (250 × 4.6 mm i.d.) at a 1.0 mL/min flow rate.[25] TLC chromatography was performed on precoated 250 $\mu$m silica gel GF glass plates (Analtech, Inc.; 5 × 10 cm). Solvents and liquid starting materials were distilled prior to use. When necessary, reactions and purifications were conducted with deoxygenated solvents, under inert gas ($N_2$), and using subdued lighting. Triethyl phosphonosenecioate was synthesized according to Iqbal et al.[35] Enol ether **2** was synthesized from 1,3-dimethoxybenzene using methods we previously described.[19]

**(2*Z*,4*E*)-4-(3′-Ethyl-2′-isopropyl-2′-cyclohexen-1′-ylidene)-3-methyl-2-butenoic Acid ((9*Z*)-5).** Zinc dust (4.20 g, 64.2 mmol) was stirred with 5% HCl (10 mL) for 2 min at room temperature. The mixture was allowed to settle, and the liquid was carefully removed by pipette. In a similar fashion the Zn was washed, under nitrogen, with water (3 × 10 mL), acetone (3 × 15 mL), and ether (2 × 8 mL). After removing residual ether under a stream of nitrogen, the flask containing the Zn dust was strongly heated with a Bunsen burner flame for 30 s. The cooled Zn dust was suspended in anhydrous dioxane (6 mL), and the stirred suspension was heated to reflux. A solution of enone **3** (1.37 g, 8.25 mmol), ethyl bromosenecioate (3.83 g, 18.5 mmol), and anhydrous dioxane (4 mL) was prepared, and a portion of this solution (1 mL) was added to the heated Zn suspension. This produced an exothermic reaction, and the remainder of the solution containing enone **3** was then added during 15 min at a rate sufficient to control reflux. The final reaction mixture was stirred at reflux for 3 h and then cooled to room temperature. Water (1.5 mL) was added, the mixture was stirred for 15 min, and ether (10 mL) was added. The mixture was filtered through a pad of Celite and the filter washed well with ether (50 mL). The filtrate was extracted with 15% HCl (30 mL), water (30 mL), 1 N NaOH (40 mL), and an additional amount of water (30 mL). The basic wash and final water wash were combined, adjusted to pH 1−2 with 15% HCl, and extracted with ether (2 × 60 mL). The organic layer was dried ($Na_2SO_4$) and concentrated under vacuum to provide yellow crystals (1.66 g, 81.1% yield): mp 101−103 °C (hexane).

**(2*Z*,4*E*)-4-(3′-Ethyl-2′-isopropyl-2′-cyclohexen-1′-ylidene)-3-methyl-2-butenal ((9*Z*)-7).** A mixture of sea sand (5 g) and activated $MnO_2$ powder (3.0 g) in anhydrous ether (10 mL) was stirred for 10 min, and the ether was evaporated by gentle warming under a stream of nitrogen. The stirred residue was then heated under $N_2$ for 30 min at 120 °C. This was cooled to room temperature, $CH_2Cl_2$ (10 mL) was added, and the mixture was cooled to 0 °C in an ice bath. A cold solution of alcohol (9*Z*)-**6** (270 mg, 1.15 mmol) in $CH_2Cl_2$ (10 mL) was added, and the mixture was stirred at 0 °C for 3.5 h. The reaction mixture was filtered through a pad of flash silica, and the filter was washed with cold 30% $CH_2Cl_2$/ether (200 mL). The filtrate was concentrated to dryness under vacuum, the residual oil was placed on a preparative TLC plate, and the plate was eluted with 2:5 ether/hexane to provide recovered alcohol (37 mg) and (9*Z*)-**7** (198 mg, 74.0% yield, 85.7% conversion based on unrecovered starting material).

**Biology. Chick Skin CRABP-Binding Assay.** Inhibition constants at 50% ($IC_{50}$) for retinoid binding to CRABP-II were determined according to a previously published method by Sani et al.[26] Briefly, CRABP-II (about 1 mg) isolated from chick embryo skin was incubated with 300 pmol of [$^3$H]-(*all-E*)-RA in the presence or absence of varying concentrations of the unlabeled retinoid (1-, 5-, 10-, 25-, and 50-fold molar excess). Free radioactively labeled retinoic acid was removed by adsorption on dextran-coated charcoal. A sucrose density gradient (5−20% sucrose) sedimentation was performed for 18 h at 180000*g*. The radioactive profiles of the 2S CRABP peak were obtained, and the $IC_{50}$ values were obtained from a probit analysis[36] of the specifically bound [$^3$H]-(*all-E*)-RA versus the log of molar concentrations of unlabeled retinoids.

**Fluorometric Determinations of Ligand−Protein Dissociation Constants.** The apparent binding affinities of the retinoid analogs for the recombinant mCRABP-I, mCRABP-II, and the ligand binding domain (DEF) of human RXRα [hRXRα (DEF)] were determined by equilibrium fluorometric titration. Proteins were expressed in *Escherichia coli* and purified to homogeneity as described by Norris et al.[27] and Cheng et al.[28] Retinoid stock solutions were made in ethanol and stored under nitrogen at −80 °C. The concentration of the stock solutions were determined by UV/vis spectroscopy from dilutions in hexane using the reported extinction coefficients. Since each retinoid exhibited significant absorbance at 330 nm, the tryptophan fluorescence is expected to be quenched by these retinoids upon binding to CRABPs and hRXRα (DEF). Fluorometric titrations were performed at 25 °C as previously described for retinoic acid by Norris et al.[27] Monochrometer slits were set to 2 and 5 nm for excitation and emission, respectively. The titration data were corrected for inner filter effects. Fluorometric titrations were initially carried out using $10^{-6}$ M protein solutions to survey the binding affinity and to determine stoichiometry for tight binders. In order to measure more accurately the apparent dissociation constants in the nanomolar or subnanomolar range, the titrations were performed at $10^{-8}$ M protein concentrations.

**Inhibition Concentrations at 50% ($IC_{50}$) for Retinoids with RARs and RXRs.** The procedure for the determination

of $IC_{50}$ values for the isomers of retinoid **1** were based on methods previously published by Allenby et al.[11,29] for (9Z)-RA and (*all-E*)-RA. Briefly, COS-1 cells were transfected with pSG5 expression plasmids containing cDNAs encoding one of the RARs or RXRs (α-, β-, or γ-subfamilies). Nucleosol fractions were prepared and stored at −80 °C. Aliquots of these fractions were incubated with assay buffer as described by Levin et al.[9a] with tritiated RA or (9Z)-RA and unlabeled ligands in ethanol. To survey the binding of test compounds to RARs and RXRs, an initial screen was performed by incubating 5 nM of [$^3$H]-(*all-E*)-RA and 1000 nM of test compound with the RAR preparation at 4 °C for 4 h or 20 nM of [$^3$H]-(9Z)-RA and 1000 nM of test compound with the RXR preparation. To determine $IC_{50}$ values, this experiment was repeated with nine concentrations of unlabeled ligand (0−10 μM) in duplicate. Free ligands were separated from the bound ligands using disposable PD-10 desalting columns (Pharmacia LKB Biotecnology Inc.) which were washed as previously described by Levin et al.[9a] Total activity, specific ligand binding, and nonspecific ligand binding were determined by scintillation counting as previously described by Allenby et al.[29] The percent of specific binding was defined as the ratio of observed binding at each concentration of competitive ligand and the specific binding. The $IC_{50}$ values were calculated by a fit of the 18 data points to the equation: $Y = [(A - D)/1 + (X/C)^B] + D$, where $Y$ is the percent specific binding, $A$ is the maximal response (set to 100%), $D$ is the minimal response (set to 0%), $C$ is the $IC_{50}$ value, and $B$ is the slope of the linear portion of the response of the log dose−response curve. The parameters $B$ and $C$ were adjusted to allow for the best fit of the data. The $IC_{50}$ values for (*all-E*)-RA binding to RARs were routinely determined, and the estimated error in these values were judged to be less than 20% of the mean.

**Transient Transfection Assays.** CV-1 cells were grown in DME medium supplemented with 10% fetal bovine serum (FBS). Transient transfection of these cells with a DNA plasmid was performed essentially as described previously by Husmann et al.[37] and Pfahl et al.[38] with minor modifications. Twenty-four hours before transfection, cells were plated at 5 × $10^4$ cells per well in a 24-well plate. Four hours before the addition of DNA−calcium phosphate precipitates, the cells were fed with DME medium supplemented with charcoal-stripped FBS. The DNA used in the transfection consisted of 100 ng of either RARα, RARβ, or RARγ and 200 ng of reporter gene $(TREpal)_2$-tk-chloramphenicol acetyltransferase (CAT), 300 ng of β-galactosidase expression plasmid pCH110, and 400 ng of carrier bluescript plasmid. When RXR homodimer or RAR/RXR heterodimer activities were investigated, 20 ng of RXRα expression vector alone or together with 25 ng of RARα expression vector were used. After overnight incubation in the presence of the DNA precipitates, cells were washed once with phosphate-buffered saline (PBS) and grown for 24 h in the medium containing 10% charcoal-stripped FBS and various concentrations of the (*all-E*)-RA or the test retinoids. At the end of the incubation, cells were washed once with PBS and lysed for 10 min at room temperature in 0.25 mL of 100 mM Tris·HCl, pH 7.8, containing 0.5% Triton X-100. The resultant cell extracts were assayed for β-galactosidase and CAT activity as described by Hussman et al.[37] The CAT activity of each individual sample was normalized for transfection efficiency by the corresponding β-galactosidase activity.

**Gel Retardation Assays.** To synthesize RXRα protein *in vitro*, DNA sequences encoding RXRα cloned into pBluescript were transcribed by using T3 RNA polymerase, and the transcripts were translated in the rabbit reticulocyte lysate system (Promega) as described previously by Zhang et al.[30] The relative amount of the translated proteins were determined by separating the $^{35}$S-methionine-labeled proteins on sodium dodecyl sulfate polyacrylamide gels, quantitating the amounts of incorporated radioactivity, and normalizing it relative to the content of methionine residues in RXR. To perform gel retardation assays, the *in vitro*-synthesized RXRα protein was incubated with $^{32}$P-labeled TREpal in a 20-μL reaction mixture containing 10 mM Hepes buffer, pH 7.9, 50 mM KCl, 1 mM DTT, 2.5 mM $MgCl_2$, 10% glycerol, and 1 μg of poly(dI−dC) at 25 °C for 20 min. The reaction mixture was loaded on a 5% nondenaturing polyacrylamide gel containing 45.5 mM Tris-borate, 45.5 mM boric acid, and 2 mM EDTA. When retinoids were used, they were incubated with RXR for 10 min at room temperature prior to performing the DNA binding assay. The TREpal oligonucleotide was labeled by Klenow DNA polymerase and the labeled oligonucleotides were purified by gel electrophoresis and used as probes for the gel retardation assay.

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