Synthesis of High Specific Activity [^{3}H]-9-*cis*-Retinoic Acid and Its Application for Identifying Retinoids with Unusual Binding Properties

Marcus F. Boehm,*,† Michael R. McClurg,‡ Charles Pathirana,† David Mangelsdorf,§ Steven K. White,† Jonathan Hebert,† David Winn,† Mark E. Goldman,⊥ and Richard A. Heyman‡

Departments of Medicinal Chemistry, Cell Biology, and New Leads Discovery, Ligand Pharmaceuticals, Incorporated, San Diego, California 92121, and Howard Hughes Medical Institute, Department of Pharmacology, University of Texas, Southwestern Medical Center, Dallas, Texas 95235

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all-trans-Retinoic acid is known to bind to the retinoic acid receptors (RARs) resulting in an increase in their transcriptional activity. In contrast, recently identified 9-*cis*-retinoic acid (9-*cis*-RA), which is an additional endogenous RA isomer, is capable of binding to both RARs and retinoid X receptors (RXRs). These distinct properties have raised questions as to the biological role governed by these two retinoic acid isomers and the set of target genes that they regulate. Herein, we report the synthesis of high specific activity [^{3}H]-9-*cis*-RA and its application to study the ligand-binding properties of the various retinoid receptor subtypes. We examined the binding properties of RARs and RXRs for a series of synthetic retinoids and compared the ligand-binding properties of these arotinoid analogs with their ability to regulate gene expression via the retinoid receptors in a cotransfection assay. The utilization of the [^{3}H]-9-*cis*-RA competitive binding assay and the cotransfection assay has made it possible to rapidly identify important structural features of retinoids leading to increased selectivity for either the RAR or RXR receptor subtypes.

Introduction

The role of retinoids such as *all-trans*-retinoic acid (ATRA), 13-*cis*-retinoic acid (13-*cis*-RA), and synthetic RA analogs in mediating cell growth and differentiation has generated interest in their pharmacological utility for treatment of dermatological diseases, such as psoriasis[1] and acne, as well as for oncological applications, such as chemotherapy and chemoprevention.[2,3] Several retinoids, among them ATRA, 13-*cis*-RA, and etretinate (Chart 1), are currently marketed for treatment of dermatological diseases and are experimentally being evaluated for cancer applications. Although these retinoids have proven therapeutically effective in the treatment of such diseases, their toxicities have limited and/or prevented their use for prolonged periods. Significant advances in elucidating the molecular basis of retinoid action now offer the potential for designing compounds with improved therapeutic indices.

Recently, receptors for retinoic acid have been identified as members of a superfamily of intracellular receptors which function as ligand-dependent transcription factors.[4,5] At present, these receptors have been classified into two subfamilies, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). The classification of these subfamilies is based primarily on differences in: (a) amino acid structure, (b) responsiveness to different naturally occurring and synthetic retinoids, and (c) ability to modulate expression of different target genes. Each RAR and RXR subfamily has three distinct subtypes designated $RAR_{\alpha,\beta,\gamma}$ and $RXR_{\alpha,\beta,\gamma}$. The discovery of multiple retinoid receptors raises questions of the functional properties of the distinct subfamilies and their receptor subtypes.

ATRA is capable of binding members of the RAR subfamily, thereby resulting in the activation of gene expression.[6,7,12] In contrast, ATRA is not capable of binding to members of the RXR subfamily, although in a cell-based cotransfection assay it has been shown to be capable of regulating gene expression via the RXRs.[7-9] This led to the speculation that ATRA was being converted to a retinoic acid metabolite that was capable of binding directly to RXR.[8] Recently, we and others discovered that 9-*cis*-RA is capable of binding and modulating gene expression via the RXRs,[9,11] suggesting that ATRA is not the only RA isomer that is biologically active. This was further supported by the isolation of 9-*cis*-RA (Ligand No. LG100057) from mammalian tissues.[9]

9-*cis*-RA binds directly to the RXRs with nanomolar affinity.[7,9,11] Interestingly, 9-*cis*-RA is also capable of binding directly to the RARs, thereby leading to the transcriptional control of RAR responsive genes.[7,12] This implies that unlike ATRA, 9-*cis*-RA is a biologically active ligand for members of the RAR and RXR subfamilies. In addition, it implies that the isomerization of retinoids is involved in controlling signal-transduction pathways and further suggests that 9-*cis*-RA may play a critical role in regulating retinoid-responsive pathways.

The discovery of a second endogenous retinoic acid isomer has led us to further investigate the biochemical properties of the retinoid receptors with the RA isomer as well as with synthetic retinoids. We have employed both a ligand-binding assay and a receptor/reporter cotransfection assay which allows us to monitor regulation of gene expression. The ligand-binding studies require high specific activity radiolabeled 9-*cis*-RA (>25 Ci/mmol), and toward this end, we report the radiochemical synthesis of isomerically pure, high specific activity [^{3}H]-9-*cis*-RA. Together with the cotransfection assay, we have applied the [^{3}H]-9-*cis*-RA competitive binding studies to identify the biological activity in a series of retinoidal benzoic acid

* Corresponding author address: Department of Medicinal Chemistry, Ligand Pharmaceuticals, Inc., 9393 Towne Centre Drive, San Diego, CA 92121.
† Department of Medicinal Chemistry.
‡ Department of Cell Biology.
§ University of Texas.
⊥ Department of New Leads Discovery.
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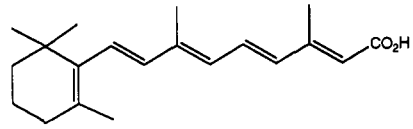
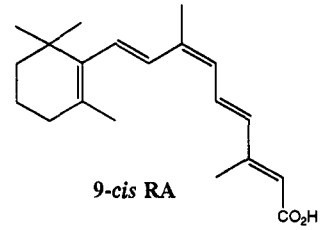
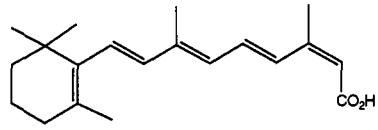
Chart 1



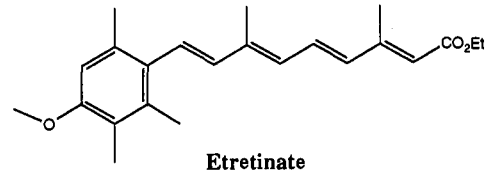
ATRA



9-*cis* RA

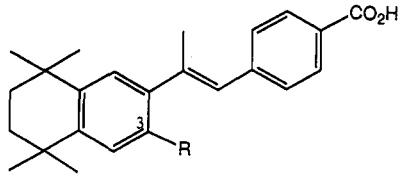


13-*cis* RA



Etretinate

Chart 2



R	
H	TTNPB
Me	3-Me-TTNPB
Et	3-Et-TTNPB
*i*Pr	3-iPr-TTNPB

derivatives (Chart 2). These data have begun to elucidate structural features of these arotinoids that determine retinoid receptor selectivities.

Chemistry

9-*cis*-Retinoic acid was synthesized from methyl (2*Z*,4*E*)-3-methyl-5-(2,6,6-trimethylcyclohex-1-enyl)penta-2,4-dienoate[13] (1) in four steps as shown in Scheme 1. Methyl ester 1 was reduced to alcohol 2 with $LiAlH_4$ at −78 °C followed by oxidation to aldehyde 3 with MnO_2. A 4:1 ratio of two isomers (2*Z*,4*E* and 2*E*,4*E*) of aldehyde 3 was observed after oxidation with MnO_2, and the mixture of isomers was directly added to the anion of diethyl [3-(ethoxycarbonyl)-2-methylprop-2-enyl]phosphonate (4)[14] at 0 °C. A mixture of two major isomers and two minor isomers (<20% of the total) of ethyl retinoate 5 was formed and directly hydrolyzed with methanolic KOH to give a mixture of retinoic acid isomers. The major isomers were identified as a 10:3 ratio of 9-*cis*- and 9,13-*dicis*-retinoic acid, and the minor isomers were a 1:1 ratio of *all-trans*- and 13-*cis*-retinoic acid. The desired major isomer, 9-*cis*-RA, was selectively crystallized from MeOH, and its ^{13}C-NMR spectra and melting point correlated with previously reported data.[15-17]

The tritium-labeled material was synthesized in an analogous manner except that carrier-free labeled $LiAl[^3H]_4$ [18,19] was used in the reduction of methyl ester 1. (A similar method for preparing labeled ATRA at low specific activity has been reported by Kaegi and DeGraw.[20]) The reduction, performed at room temperature, gave a 1:1 mixture of 2*Z*,4*E* and 2*E*,4*E* isomers of alcohol **2a**. Oxidation of **2a** with MnO_2 afforded aldehyde **3a**, which was passed through a 1-cm pad of silica gel to remove all water before addition to the diethyl-[3-(ethoxycarbonyl)-2-methylprop-2-enyl]phosphonyl anion 4 at 0 °C. Ester **5a** was hydrolyzed as above, and the resulting mixture of isomers was identified as a 3:3:1:1 mixture of 9-*cis*-:*all-trans*-:9,13-*dicis*-:13-*cis*-retinoic acid by ODS HPLC. The mixture was separated by semipreparative ODS HPLC to give pure [^{3}H]-9-*cis*-**6a** and *all-trans*-retinoic acid at a specific activity of 29 Ci/mMol.

The (*E*)-4-[2-(5,6,7,8-tetrahydro-3-alkyl-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (3-alkyl-TTNPB) derivatives (Chart 2) were synthesized by the methods previously described.[21-24]

Biological Studies

The properties of synthetic retinoids were examined in two separate assays: a competitive binding assay with [^{3}H]-9-*cis*-retinoic acid[9,12] and a cotransfection assay in CV-1 cells.[8,25,26]

The ligand-binding properties of the naturally occurring retinoic acid isomers were examined employing a competitive binding assay with [^{3}H]-9-*cis*-RA. Binding studies demonstrate that both ATRA and 9-*cis*-RA compete with [^{3}H]-9-*cis*-RA binding to all members of the RAR subfamily (Table 1). 9-*cis*-RA has equilibrium binding constants of 7, 7, and 17 nM for the $RAR_{\alpha,\beta,\gamma}$, respectively, whereas ATRA has equilibrium binding constants of 15, 13, and 18 nM for the $RAR_{\alpha,\beta,\gamma}$, respectively. As previously reported, only 9-*cis*-RA binds to RXRs and has binding constants of 32, 12, and 4 nM for the $RXR_{\alpha,\beta,\gamma}$, respectively. Therefore, the use of [^{3}H]-9-*cis*-RA allows us to examine the ligand-binding properties of compounds that bind to members of both the RAR and RXR subfamilies.

In addition to ATRA and 9-*cis*-RA, we examined the ligand-binding properties of the arotinoid TTNPB and the 3-methyl derivative of TTNPB (Chart 2). Both TTNPB and 3-methyl-TTNPB are capable of competing with [^{3}H]-9-*cis*-RA at RAR_α in a concentration-dependent fashion with IC_{50} values of 36 and 638 nM, respectively (Table 1). Both compounds also bind to RAR_β and RAR_γ. In contrast, only 3-methyl-TTNPB competes with 9-*cis*-RA at the RXRs with IC_{50} values of 32 and 100 nM for $RXR_{\alpha,\gamma}$, respectively. These data imply that an alkyl substituent in the 3-position of TTNPB results in an

Scheme 1

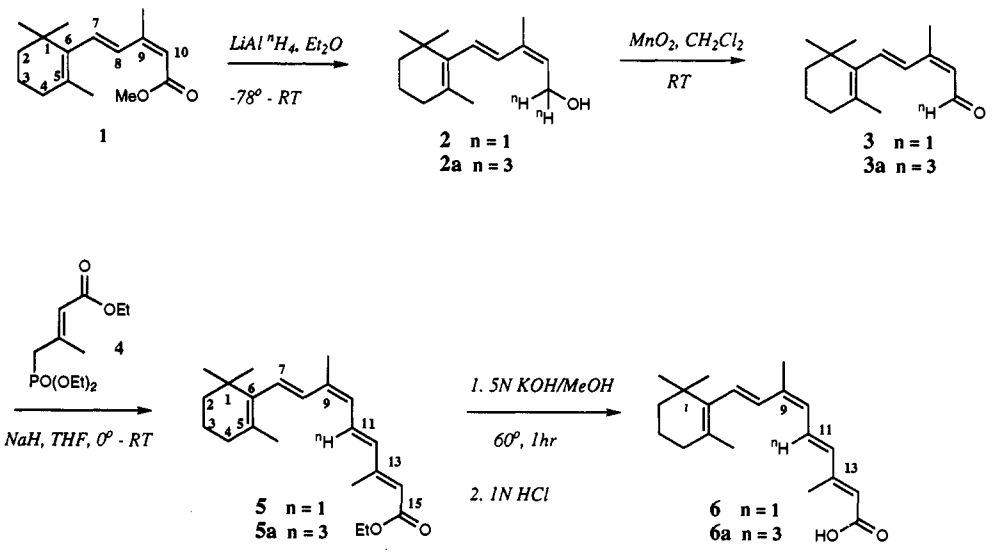


Table 1. Competition of [^{3}H]-9-*cis*-Retinoic Acid from Retinoic Acid and Retinoid X Receptors

	IC_{50} (nM)[a]					
compound	RAR_α	RAR_β	RAR_γ	RXR_α	RXR_β	RXR_γ
ATRA	15 ± 2.0	13 ± 3.0	18 ± 1.8	>1000	>1000	350 ± 40
9-*cis*-RA	7 ± 1.7	7 ± 1.3	17 ± 1.1	32 ± 3.5	12 ± 3.0	4 ± 2.0
TTNPB	36 ± 5.1	5 ± 2.3	26 ± 4.3	>1000	>1000	>1000
3-methyl-TTNPB	638 ± 75	1169 ± 274	645 ± 120	32 ± 8.0	>1000	100 ± 23
3-ethyl-TTNPB	100 ± 49	75 ± 20	250 ± 38	320 ± 40	50 ± 10	10 ± 6.0
3-isopropyl-TTNPB	>1000	150 ± 42	>1000	320 ± 65	50 ± 8.0	10 ± 4.0

[a] RA and RX receptors were prepared from Baculo Sf21 extracts. Values are the mean ± standard error of the mean, based on three determinations.

Table 2. Cotransfection Data from CV-1 Cells

	EC_{50} (nM)[a]					
compound	RAR_α	RAR_β	RAR_γ	RXR_α	RXR_β	RXR_γ
ATRA	350 ± 31	80 ± 9.0	10 ± 2.0	900 ± 70	1400 ± 130	1100 ± 85
9-*cis*-RA	191 ± 20	50 ± 17	45 ± 5.0	100 ± 25	200 ± 30	140 ± 13
TTNPB	30 ± 6.0	3 ± 2.0	2 ± 1.0	>10 000	>10 000	>10 000
3-methyl-TTNPB	340 ± 30	230 ± 28	180 ± 15	1200 ± 120	1175 ± 150	1500 ± 111
3-ethyl-TTNPB	1700 ± 118	310 ± 29	23 ± 4.0	2900 ± 250	2000 ± 150	2600 ± 160
3-isopropyl-TTNPB	>10 000	>10 000	270 ± 35	300 ± 32	>10 000	2700 ± 240

[a] Values are the mean ± standard error of the mean, based on six determinations.

enhancement in the affinity for RXR and decreases the affinity for RAR.

Furthermore, the desmethyl derivative selectively binds to the RAR isoforms. To further distinguish the structural determinants that alter RAR and RXR activity, we prepared other TTNPB derivatives with alkyl substituents at the 3-position, including the ethyl and isopropyl derivatives. The ethyl and isopropyl derivatives resulted in a loss of RAR-binding activity with the isopropyl derivative being virtually devoid of activity at RAR_α and RAR_γ (Table 1). In contrast, these compounds show some ability to bind to members of the RXR subfamily.

The biological properties of the RA isomers and the arotinoids were further characterized in a cotransfection assay by examining their ability to interact with retinoid receptors resulting in a modulation of gene expression. The six retinoic acid receptor subtypes were individually cotransfected into CV-1 cells along with a reporter molecule that contained a retinoid receptor-response element. This allows one to examine the ability of the retinoids to modulate gene expression via their respective retinoid receptor isoforms upon addition of the appropriate ligand.

ATRA is a good activator of $RAR_{\alpha,\beta,\gamma}$ with EC_{50} values of 350, 80, and 10 nM, respectively; however, it is less active at $RXR_{\alpha,\beta,\gamma}$ with EC_{50} values of 900, 1400, and 1100 nM, respectively (Table 2). In contrast, 9-*cis*-RA is a potent activator of both $RAR_{\alpha,\beta,\gamma}$ (EC_{50} values of 191, 50, and 45 nM, respectively) and $RXR_{\alpha,\beta,\gamma}$ (EC_{50} values of 100, 200, and 140 nM, respectively).

TTNPB has previously been shown to selectively activate members of the RAR subfamily.[8] Indeed, TTNPB functions as a potent activator of $RAR_{\alpha,\beta,\gamma}$ but does not activate $RXR_{\alpha,\beta,\gamma}$ in our cotransfection assay (Table 2, Figure 1A). Examination of 3-alkyl-substituted TTNPB derivatives shows a different activation profile: in the cotransfection assay, 3-methyl-TTNPB is a (10–200)-fold weaker activator of the RARs than is its parent TTNPB (shown for RAR_β in Figure 1B). In contrast, 3-methyl-TTNPB activates all members of the RXR subfamily as well as all members of the RAR subfamily (Table 2). Comparison of the transactivation properties of TTNPB and 3-methyl-TTNPB on the RAR and RXR subtypes reveals that substitution at the 3-position results in a shift in activity toward a more balanced retinoid receptor activator (Table 2). Finally, results from cotransfection assays with 3-ethyl- and 3-isopropyl-TTNPB showed

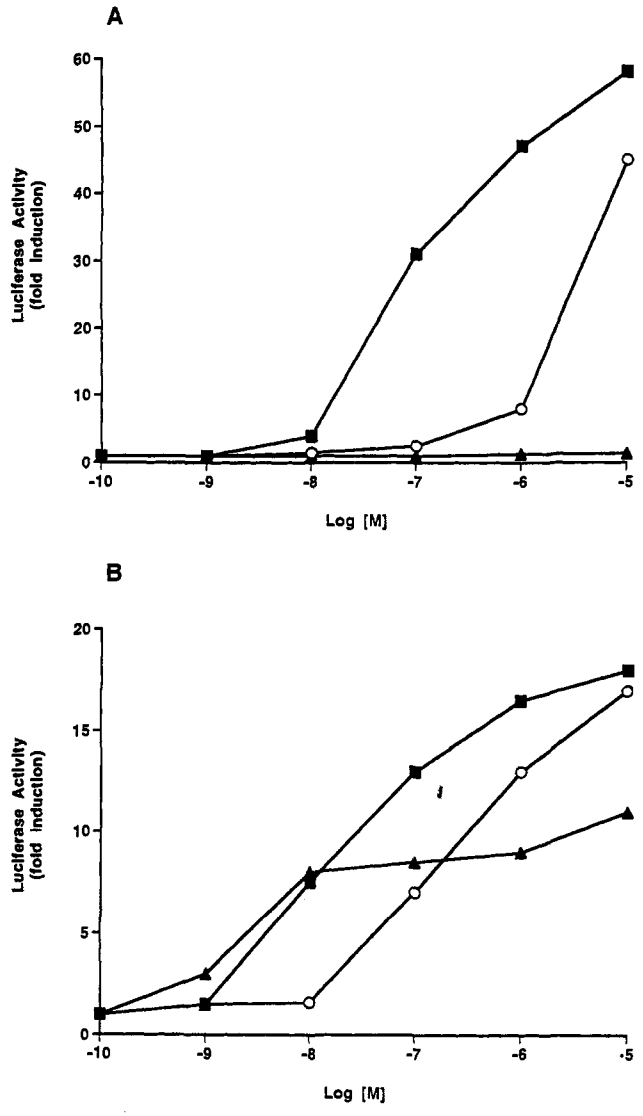


Figure 1. Retinoid-induced transactivation; rank order potency of retinoids on RXR_α and RAR_β. (A) Comparison of RXR_α transactivation by 9-*cis*-RA, TTNPB, and 3-methyl-TTNPB. CV-1 cells were cotransfected with the RXR_α expression vector and the luciferase reporter plasmid containing a retinoid X receptor-response element (RXRE) consisting of CRBPII-tk-Luc. The cells were incubated with increasing concentrations of 9-*cis*-RA (squares), TTNPB (triangles), or 3-methyl-TTNPB (open circles). Transactivation is expressed as induction of luciferase activity (X control levels) with retinoid-treated cells compared with solvent-treated control cells and represents the mean of four separate assays. (B) Comparison of RAR_β transactivation by 9-*cis*-RA, TTNPB, and 3-methyl-TTNPB. CV-1 cells were cotransfected with the RAR_β expression vector and a retinoid-response element containing the luciferase reporter molecule, TREp-ΔMTV-Luc. Cells were then treated with the retinoids shown in (A). Data are expressed as in (A).

similar, albeit lower, activation profiles across all six receptors as 3-methyl-TTNPB, as was observed in the binding studies.

Thus, the competitive binding assay data using [^{3}H]-9-*cis*-RA and the cotransfection assay data for TTNPB and 3-alkyl-TTNPB derivatives indicate that alkyl substituents in the 3-position of TTNPB significantly reduce the RAR selectivity of TTNPB and produce a ligand which activates members of both the RXR and RAR isoforms.

Discussion and Conclusion

In the course of this study, an important biochemical tool was developed, namely [^{3}H]-9-*cis*-RA, to examine the ligand-binding properties of retinoids at their putative molecular targets, the RARs and RXRs. The utility of high specific activity [^{3}H]-9-*cis*-RA is apparent in competitive binding studies for the purpose of determining structure–activity relationships of synthetic retinoids. Since 9-*cis*-RA is capable of binding all six retinoid receptor subtypes with comparable IC_{50} values, it is often referred to as a "pan-agonist". The pan-agonist properties of this isomer are useful for competitive binding assay of synthetic retinoids with respect to all six retinoid receptor subtypes. In addition, we have employed a cotransfection assay system that has allowed us to examine the ability of synthetic retinoids to interact with the different retinoid receptors, thereby regulating gene expression. We chose to focus on the arotinoids TTNPB and 3-methyl-TTNPB, based on the observation that TTNPB and its 3-methyl derivative had distinct biological responses on F9 and HL-60 differentiation.[24,27] Specifically, TTNPB was more potent than 3-methyl-TTNPB in inducing F9 cell differentiation, whereas the reciprocal activity was observed in HL-60 differentiation. Furthermore, it was reported that these two related compounds had distinctly different teratogenic profiles.[28-30] We observed that addition of an alkyl moiety to the 3-position of TTNPB significantly altered the binding and biological activity of TTNPB, allowing binding and activation of RXR receptors. The biological differences observed by Davies,[27] Strickland,[24] and Kissler[28-30] may now, in part, be attributed to their distinctly different ligand-binding activity and their different ability to regulate gene expression among the six retinoid receptors.

The results of the above study demonstrate that the ability to conduct both ligand binding and gene expression provides valuable tools to identify compounds with unique receptor selectivities. The structure–activity relationship observed for 3-alkyl-TTNPB analogs should provide an opportunity to develop novel compounds with unique pharmacological properties.

Experimental Section

Unless otherwise stated, all reactions were carried out under a nitrogen atmosphere. The organic solvents were purchased from Fisher Scientific, TLC was performed with Merck Kieselgel 60 F-254 plates, and ^{1}H-NMR and ^{3}H-NMR spectra were determined on Bruker 300 and 400 MHz spectrometers (protons and tritons are assigned by the accepted retinoid numbering system as shown in Scheme 1). UV spectra were measured on a Kontron Uvikon Model 941 spectrometer, HPLC purification was performed on a Waters system using a Beckman C_{18} Ultrasphere (5-μm, 10-mm × 25-cm) column, and mass spectra were recorded on a Hewlett-Packard GCMS Model 5890 mass spectrometer.

(2*Z*,4*E*)-3-Methyl-5-(2,6,6-trimethylcyclohex-1-enyl)penta-2,4-dien-1-ol (2). To 40 mg (0.16 mmol) of methyl (2*Z*,4*E*)-3-methyl-5-(2,6,6-trimethylcyclohex-1-enyl)penta-2,4-dienoate[12] (1) in 2 mL of anhydrous THF at −78 °C was added 0.25 mmol of $LiAlH_4$. The reaction mixture was stirred at −78 °C for 15 min and warmed to room temperature. When the reaction was complete (monitored by TLC), 2 mL of water was added and the organics were extracted with ether and washed three times with 5 mL of water. The organic layer was dried with $MgSO_4$, filtered, and concentrated *in vacuo*, and dienol 2 was used directly in the next step: R_f (20% EtOAc–hexane) = 0.26 (cis), 0.20 (trans); ^{1}H NMR (cis) ($CDCl_3$) δ 1.01 (s, 1-(CH_3)$_2$), 1.46 (t, J = 6 Hz, 2-CH_2), 1.62 (dt, J = 6 Hz, J = 4 Hz, 3-CH_2), 1.70 (s, 5-CH_3), 1.91 (s, 9-CH_3), 2.01 (t, J = 6 Hz, 4-CH_2), 4.30 (d, J = 6 Hz, 11-CH_2), 5.54 (t, J = 6 Hz, 10-C*H*), 6.19 (d, J = 16 Hz, 7-C*H*), 6.39 (d, J = 16 Hz, 8-C*H*).

(2*Z*,4*E*)-3-Methyl-5-(2,6,6-trimethylcyclohex-1-enyl)penta-2,4-dien-1-al (3). To the crude dienol 2 in 5 mL of CH_2Cl_2

was added 100 mg (1.1 mmol) of MnO_2. The reaction mixture was stirred vigorously for 1 h at room temperature or until the reaction was complete by TLC. The product had a distinct yellow/brown color upon heating a vanillin-stained TLC plate. Two products were observed by TLC and identified as 2*E*:4*Z* and 2*E*:4*E* isomers in ca. 5:1 ratio by ^{1}H NMR. After completion, the product was filtered through Celite and the celite washed with CH_2Cl_2 (3 × 15 mL). The product was concentrated, and aldehyde **3** was used directly in the next step: R_f (20% EtOAc–hexane) = 0.55 (cis), 0.47 (trans); ^{1}H NMR (cis) δ ($CDCl_3$) 1.06 (s, 1-(CH_3)$_2$), 1.50 (t, J = 2 Hz, 4-CH_2), 1.63 (dt, J = 6 Hz, J = 2 Hz, 3-CH_2), 1.75 (s, 5-CH_3), 2.06 (t, J = 6 Hz, 2-CH_2), 2.13 (s, 9-CH_3), 5.87 (d, J = 8 Hz, 10-C*H*), 6.63 (d, J = 16, Hz, 8-C*H*), 7.09 (d, J = 16 Hz, 7-C*H*), 10.17 (d, J = 8 Hz, 11-C *H*).

Ethyl (2*E*,4*E*,6*Z*,8*E*)-3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoate (5) (Ethyl 9-*cis*-retinoate). To 40 mg (0.15 mmol) of phosphonate **4** in 3 mL of dry THF was added 7 mg (0.18 mmol) of sodium hydride (60% wt in oil). The reaction was stirred at room temperature for 0.5 h and cooled to 0 °C followed by addition of aldehyde **3** in 2 mL of THF. The reaction mixture was warmed to room temperature and stirred for an additional 30 min followed by addition of 3 mL of water. The organics were extracted with ether (3 × 5 mL), dried ($MgSO_4$), filtered, and concentrated *in vacuo*, and the crude ester **5** was used directly in the next step: R_f (20% EtOAc–hexane) = 0.79; ^{1}H NMR δ ($CDCl_3$) 1.04 (s, (CH_3)$_2$), 1.27 (t, J = 8 Hz, CH_2CH_3), 1.48 (t, J = 3 Hz, 2-CH_2), 1.64 (tt, J = 6 Hz, J = 3 Hz, 3-CH_2), 1.75 (s, 18-CH_3), 2.01 (s, 19-CH_3), 2.05 (t, J = 6 Hz, 4-CH_2), 2.35 (s, 20-CH_3), 4.15 (q, J = 8 Hz, CH_2CH_3), 5.78 (s, 14-C*H*), 6.05 (d, J = 11.5 Hz, 10-C*H* or 12-C*H*), 6.25 (d, J = 15 Hz, 10-C*H* or 12-C*H*), 6.29 (d, J = 16.5 Hz, 8-C*H*), 6.62 (d, J = 16.5 Hz, 7-C*H*), 7.06 (dd, J = 11.5 Hz, J = 15 Hz, 11-C*H*).

(2*E*,4*E*,6*Z*,8*E*)-3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoic Acid (6) (9-*cis*-Retinoic acid, LG100057). To ester **5** in 5 mL of MeOH was added 0.5 mL of 5 N KOH, and the reaction mixture was heated to 60 °C for 1 h. After the hydrolysis was complete (by TLC), the solution was cooled to 0 °C and acidified with 1 N HCl. The organics were extracted with ether (3 × 10 mL), dried ($MgSO_4$), and concentrated *in vacuo*. The product was purified by crystallization from MeOH to give 8 mg (0.027 mmol) of pure 9-*cis*-retinoic acid **6** (17% overall yield from methyl ester **1**): R_f (20% EtOAc–hexane) = 0.26; mp = 189 °C, lit.[15,16] mp 189–191 °C; UV_{MeOH} = 343 nm (ε = 39 000); ^{1}H NMR (CD_3OD) δ 1.05 (s, 1-(CH_3)$_2$), 1.52 (t, J = 3 Hz, 2-CH_2), 1.65 (tt, J = 6 Hz, J = 3 Hz, 3-CH_2), 1.75 (s, 18-CH_3), 2.00 (s, 19-CH_3), 2.06 (t, J = 6 Hz, 4-CH_2), 2.29 (s, 20-CH_3), 5.79 (s, 14-CH), 6.11 (d, J = 12 Hz, 10-C*H*), 6.30 (br d, J = 16 Hz, 7-C*H* + 12-C*H*), 6.70 (d, J = 17 Hz, 8-CH), 7.11 (dd, J = 12 Hz, J = 15 Hz, 11-C*H*); MS (FAB$^+$) found 300.20867, calcd 300.20892; ^{13}C NMR δ ($CDCl_3$) 14.1, 19.2, 20.9, 21.9, 28.9, 28.9, 33.0, 34.2, 39.5, 117.7, 127.9, 129.4, 130.2, 130.5, 130.6, 134.2, 138.0, 139.1, 155.3, 172.5.

Synthesis of [^{3}H]-9-*cis*-RA. In order to minimize radiation-induced degradation due to the large quantity of radioactivity (>5 Ci), the whole synthetic sequence and the purification were performed in 1 day. The syntheses of **2a**–**3a** and **5a** were performed in a glovebox. Synthesis of **6a** and the HPLC purification were performed in a fume hood. The final products were stored under dilute conditions (1 mCi/mL in 1:1 EtOH–toluene) at −78 °C. Under these storage conditions, it was determined by HPLC that the labeled retinoic isomers exhibited less than 3% isomerization over 6 months.

[1,1-^{3}H]-(2*Z*,4*E*)-3-Methyl-5-(2,6,6-trimethylcyclohex-1-enyl)penta-2,4-dien-1-ol (2a). To 0.25 mmol of $LiAl[^3H_4]$[13,14] in 1 mL of THF at −78 °C was added 40 mg (0.16 mmol) of methyl ester **1**[12] in 2 mL of anhydrous THF. The reaction mixture was stirred at −78 °C for 15 min and warmed to room temperature for 6 h. When the reaction was ca. 40% complete (monitored by TLC), 2 mL of MeOH was added and the volatile components were removed *in vacuo*. The organics were resuspended in ether and washed three times with 5 mL of water. The organic layer was dried with $MgSO_4$, filtered, and concentrated *in vacuo*, and the product alcohol **2a** was used directly in the next step: R_f (20% EtOAc–hexane) = 0.26 (cis), 0.20 (trans).

[1-^{3}H]-(2*Z*,4*E*)-3-Methyl-5-(2,6,6-trimethylcyclohex-1-enyl)-penta-2,4-dien-1-al (3a). To the crude alcohol **2a** in 5 mL of CH_2Cl_2 was added 100 mg (1.1 mmol) of MnO_2. The reaction mixture was stirred vigorously at room temperature until the reaction was complete by TLC. The product had a distinct yellow/brown color upon heating a vanillin-stained TLC plate. Two products in ca. 1:1 ratio were observed by TLC and identified as *E*:*E* and *E*:*Z*. After the oxidation reaction was complete (by TLC), the product was filtered through Celite and the Celite washed three times with CH_2Cl_2. The product was concentrated, and aldehyde **3a** was used directly in the next step: R_f (20% EtOAc–hexane) = 0.55 (cis), 0.47 (trans).

[5-^{3}H]-Ethyl (2*E*,4*E*,6*Z*,8*E*)-3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoate (5a) ([^{3}H]-Ethyl 9-*cis*-retinoate). To 40 mg (0.15 mmol) of phosphonate **4** in 3 mL of dry THF was added 7 mg (0.18 mmol) of sodium hydride (60% in oil). The reaction mixture was stirred at room temperature for 0.5 h and cooled to 0 °C followed by addition of aldehyde **3a** in 2 mL of THF. The reaction mixture was warmed to room temperature and stirred for an additional 30 min. Water was added (3 mL), and the organics were extracted with ether (3 × 5 mL), dried ($MgSO_4$), filtered, and concentrated (rotoevaporator). The crude ester **5** was used directly in the next step: R_f (20% EtOAc–hexane) = 0.79.

[5-^{3}H]-(2*E*,4*E*,6*Z*,8*E*)-3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoic Acid (6a) ([^{3}H]-9-*cis*-Retinoic acid). To ester **5a** in 5 mL of MeOH was added 0.5 mL of 5 N KOH, and the reaction mixture was heated to 60 °C for 1 h. After the hydrolysis was complete (by TLC), the solution was cooled to 0 °C and acidified with 1 N HCl. The organics were extracted with ether (3 × 10 mL), dried ($MgSO_4$), and concentrated *in vacuo*. The product acid **6a** was purified by ODS HPLC to give 110 mCi of 9-*cis*-retinoic acid, 80 mCi of *all-trans*-retinoic acid, 15 mCi of 13-*cis*-retinoic acid, and 20 mCi of 9,13-*dicis*-retinoic acid. The specific activity was ca. 29 Ci/mmol and was determined by ^{3}H NMR and ^{1}H NMR which showed only tritide and no detectable hydride at the 11-position of 9-*cis*-RA. The radiolabeled retinoic acid was stored at a concentration of 1 mCi/mL in 1:1 EtOH–toluene at −78 °C in order to reduce radiation-induced decomposition. **6a**: R_f (20% EtOAc–hexane) = 0.26; UV_{MeOH} = 343 nm; ^{1}H NMR (CD_3OD) δ 1.05 (s, 1-(CH_3)$_2$), 1.52 (t, J = 3 Hz, 2-CH_2), 1.65 (tt, J = 6 Hz, J = 3 Hz, 3-CH_2), 1.75 (s, 18-CH_3), 2.00 (s, 19-CH_3), 2.06 (t, J = 6 Hz, 4-CH_2), 2.29 (s, 20-CH_3), 5.79 (s, 14-CH), 6.11 (d, J = 12 Hz, 10-C*H*), 6.30 (br d, J = 16 Hz, 7-C*H* + 12-C*H*), 6.70 (d, J = 17 Hz, 8-CH), 7.11 (dd, J = 12 Hz, J = 16 Hz, 11-C*H*); ^{3}H NMR (C D_3OD) δ 7.11 (dd, J = 12 Hz, J = 16 Hz, 11-C^3*H*).

Synthesis of TTNPB Derivatives. TTNPB derivatives may be synthesized in the same manner as described by Loeliger[21,23] and Frickel.[22]

(*E*)-4-[2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic Acid (TTNPB). The synthesis is described in detail by Loeliger:[23] mp = 242–244 °C; ^{1}H NMR δ ($CDCl_3$) 1.31 (s, (CH_3)$_2$), 1.34 (s, (CH_3)$_2$), 1.72 (s, (CH_2)$_2$), 2.31 (d, J = 1 Hz, CH_3), 6.83 (s, CH), 7.32 (m, 2-Ar-CH), 7.46 (m, Ar-CH + 2-Ar-CH), 8.10 (d, J = 8 Hz, 2-Ar-CH).

(*E*)-4-[2-(5,6,7,8-Tetrahydro-3,5,5,8,8-pentamethyl-2-naphthalenyl)-1-propenyl]benzoic Acid (3-Methyl-TTNPB). It was synthesized according to the method described by Loeliger[23] except that 5,6,7,8-tetrahydro-3,5,5,8,8-pentamethyl-2-naphthyl methyl ketone[31] was used instead of 5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl methyl ketone as starting material. (*E*)-4-[2-(5,6,7,8-Tetrahydro-3,5,5,8,8-pentamethyl-2-naphthalenyl)-1-propenyl]benzoic acid (3-methyl-TTNPB) was crystallized from EtOAc–hexane as one isomer: mp = 211–213 °C; ^{1}H NMR δ ($CDCl_3$) 1.31 (s, (CH_3)$_2$), 1.32 (s, (CH_3)$_2$), 1.71 (s, (CH_2)$_2$), 2.25 (s, CH_3), 2.32 (s, Ar-CH_3), 6.45 (s, CH), 7.13 (s, Ar-CH), 7.14 (s, Ar-CH), 7.50 (d, J = 8 Hz, 2-Ar-CH), 8.14 (d, J = 8 Hz, 2-Ar-CH).

(*E*)-4-[2-(5,6,7,8-Tetrahydro-3-ethyl-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic Acid (3-Ethyl-TTNPB). It was synthesized according to the method described by Loeliger[23] except that 5,6,7,8-tetrahydro-3-ethyl-5,5,8,8-tetramethyl-2-naphthyl methyl ketone[31] was used instead of 5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl methyl ketone as starting material. (*E*)-4-[2-(5,6,7,8-tetrahydro-3-ethyl-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (3-ethyl-TTNPB) was crystallized from EtOAc–hexane as one isomer: mp = 160–162 °C; ^{1}H NMR δ ($CDCl_3$) 1.25 (t, J = 8 Hz, –CH_2CH_3), 1.31 (s,

(CH_3)$_2$), 1.32 (s, (CH_3)$_2$), 1.71 (s, (CH_2)$_2$), 2.25 (s, CH_3), 2.32 (s, Ar-CH_3), 2.66 (q, J = 8 Hz, -CH_2CH_3), 6.45 (s, CH), 7.10 (s, Ar-CH), 7.18 (s, Ar-CH), 7.49 (d, J = 8 Hz, 2-Ar-C H), 8.14 (d, J = 8 Hz, 2-Ar-CH).

(*E*)-4-[2-(5,6,7,8-Tetrahydro-3-isopropyl-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic Acid (3-Isopropyl-TTNPB). It was synthesized according to the method described by Loeliger[23] and above the 3-ethyl-TTNPB except that 5,6,7,8-tetrahydro-3-isopropyl-5,5,8,8-tetramethyl-2-naphthyl methyl ketone[31] was used as starting material. (*E*)-4-[2-(5,6,7,8-Tetrahydro-3-isopropyl-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (3-isopropyl-TTNPB) was crystallized from EtOAc–hexane as one isomer: mp = 242–244 °C; ^{1}H NMR δ ($CDCl_3$) 1.24 (d, J = 7 Hz, CH(CH_3)$_2$), 1.29 (s, (CH_3)$_2$), 1.31 (s, (CH_3)$_2$), 1.69 (s, (CH_2)$_2$), 2.23 (s, CH_3), 2.32 (s, Ar-CH_3), 3.09 (q, J = 7 Hz, CH(CH_3)$_2$), 6.41 (s, CH), 7.04 (s, Ar-CH), 7.23 (s, Ar-CH), 7.48 (d, J = 8 H z, 2-Ar-CH), 8.12 (d, J = 8 Hz, 2-Ar-CH).

Biology. The receptor expression vectors pRS-hRAR$_\alpha$ and pRs-hRXR$_\alpha$ used in the cotransfection assay have been described previously.[8,32] A basal reporter plasmid, MTV-LUC, containing two copies of the TRE-palindromic response element MTV-TREp-LUC[26] was used in all transfections for RAR$_\alpha$. The reporter plasmid CRBPII-tk-LUC containing the RXRE from CRBPII[33] was used with the RXR$_\alpha$ transfections.

CV-1 transfections were performed as described[8,33] and modified for automation in 96-well plates.[25] Briefly, the plasmids were transiently transfected by the calcium phosphate method by using 10 ng of a receptor-expression plasmid vector, 50 ng of the reporter luciferase (LUC) plasmid, 50 ng of pRS-βGAL (β-galactosidase) as an internal control, and 90 ng of carrier plasmid pGEM. Cells were transfected for 6 h and then washed to remove the precipitate. The cells were incubated for 40 h with or without retinoid. All of the transfections were performed on a Beckman Biomek automated workstation, and cell extracts were then prepared and assayed for luciferase and β-galactosidase as described by Berger *et al.*[25] All determinations were performed in triplicate in at least two independent experiments and normalized for transfection efficiency by using β-galactosidase as the internal control.

For binding studies, retinoid receptors were used, employing a baculovirus expression system.[9,12] The methods concerning growth, purification, and assays of recombinant viruses followed the protocol outlined by Summers and Smith.[34] The recombinant plasmids were cotransfected into Sf21 cells with wild-type AcNPV DNA, and the recombinant viruses were plaque purified. For the mock (control) extracts, wild-type AcNPV-infected cells were used.

For ligand-binding assays, the baculovirus-infected cells were disrupted by Dounce homogenization (Kontes Co., Vineland, NJ) in 10 nM Tris (pH 7.6), 5 mM dithiothreitol (DTT), 2 mM EDTA, 0.5% CHAPS, and 1 mM phenylmethanesulfonyl fluoride. The KCl concentration was adjusted to 0.4 M after cell lysis. The cell lysates were centrifuged for 1 h at 4 °C, 100 000 g, and the supernatant was recovered as a high-salt, whole cell extract. For the saturation binding analysis, cell extracts (50 μg of protein) were incubated at 0 °C for 2.0 h with [^{3}H]-labeled retinoid in the presence or absence of 200-fold excess unlabeled ligand. Specific ligand binding to receptor was determined by a hydroxyapatite-binding assay according to the protocol of Wecksler and Norman.[35]

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