Syntheses of High Specific Activity 2,3- and 3,4-[^{3}H]$_2$-9-*cis*-Retinoic Acid†

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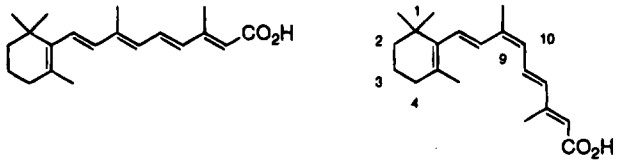
9-*cis*-Retinoic acid (9-*cis*-RA) is an endogenous hormone which binds and activates the retinoic acid receptors (RARs) and the retinoic X receptors (RXRs). In order to investigate the function of 9-*cis*-RA *in vitro* and *in vivo* high specific activity labeled 9-*cis*-RA was prepared. Two tritium labels were efficiently introduced at the 2,3- or 3,4-positions, respectively, in the cyclohexene ring moiety resulting in labeled 9-*cis*-RA with specific activity of 58–60 Ci/mmol . The critical ring-labeling step relies on a highly regioselective tritiation of either a terminal or an isolated double bond in the presence of the conjugated retinoate side chain. Moreover, the labeling is performed at the penultimate synthetic step resulting in optimization of radiochemical yields and ease of synthesis. This is the first reported synthesis of ring-labeled [^{3}H]$_2$-9-*cis*-RA, and the methodology described herein is applicable to the synthesis of other retinoic acid isomers.

Introduction

Retinoids have shown activity against a variety of cancers in laboratory animals and utility for the treatment of dermatological diseases in humans.[1] Several retinoids, including *all-trans*-retinoic acid (ATRA, **1**), Figure 1, 13-*cis*-retinoic acid (13-*cis*-RA), and etretinate, an aromatic polyenic ethyl ester, are currently in clinical trials for various chemotherapeutic cancer applications.[2] The retinoids exhibit their anticancer action by inhibiting cellular proliferation and inducing differentiation of cells through modulation of their intracellular retinoid receptors. These receptors comprise two distinct classes, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), which differ principally in their structure, responsiveness to various retinoids, and modulation of gene expression. Each class has three distinct subtypes designated as RAR α,β,γ and RXR α,β,γ.[3]

Recently, 9-*cis*-retinoic acid (9-*cis*-RA, **2**) was identified as a novel endogenous hormone in mammalian tissues.[4] Unlike ATRA which binds more specifically to the RAR's,[5] 9-*cis*-RA binds to all six receptor isoforms.[4,6] This "pan-agonist" is thus a useful probe for investigating the receptor–ligand interactions of retinoids at all six receptor subtypes. In order to investigate the biochemical properties of 9-*cis*-RA (LGD1057), which is currently in clinical trials for the treatment of various cancers, it was necessary to obtain substantial quantities (2–3 Ci) of high specific activity radiolabeled 9-*cis*-RA for competitive binding assays, bioavailability, stability, and metabolism studies.[7–10] Previously, 9-*cis* RA was synthesized with one or more tritium labels incorporated in the olefinic chain at the 11 and/or 12 positions. The synthetic methodology for preparing these radiolabeled compounds has included tritiation of a synthetically advanced, trienoic ester using a lithium aluminum tritide reduction, followed by three additional steps to give the 11-[^{3}H] 9-*cis*-RA.[8] In another report, photochemical isomerization of commercially available 11,12-[^{3}H]$_2$-ATRA, followed by HPLC isolation, gave the corresponding labeled 9-*cis* RA isomer in low yield.[9] However, in our hands *in vivo* metabolism studies with chain-labeled retinoic acids resulted in loss of significant quantities of tritium through oxidative cleavage of the polyenic chain.[10] In order to maximize the retention of the tritium label in such studies, ring-radiolabeled 9-*cis*-RA was prepared.



all-trans-Retinoic Acid (ATRA) **1** 9-*cis*-Retinoic Acid (9-*cis*-RA) **2**

* The traditional retinoids numbering system is used

Figure 1.

Herein are described two radiochemical syntheses of isomerically pure ring [^{3}H]$_2$-9-*cis*-RA with high specific activity (58–60 Ci/mmol). The radionuclide is incorporated at the penultimate synthetic step at either the 2,3- or 3,4-positions of the cyclohexenyl moiety, Figure 1. The traditional retinoid numbering is used as shown in Figure 1.

†Dedicated to Professor Stephen Hanessian on the occasion of his 60th birthday.

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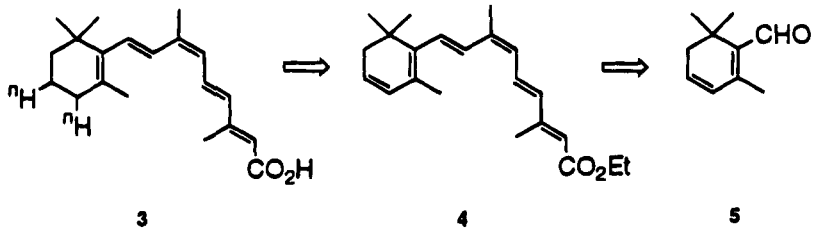
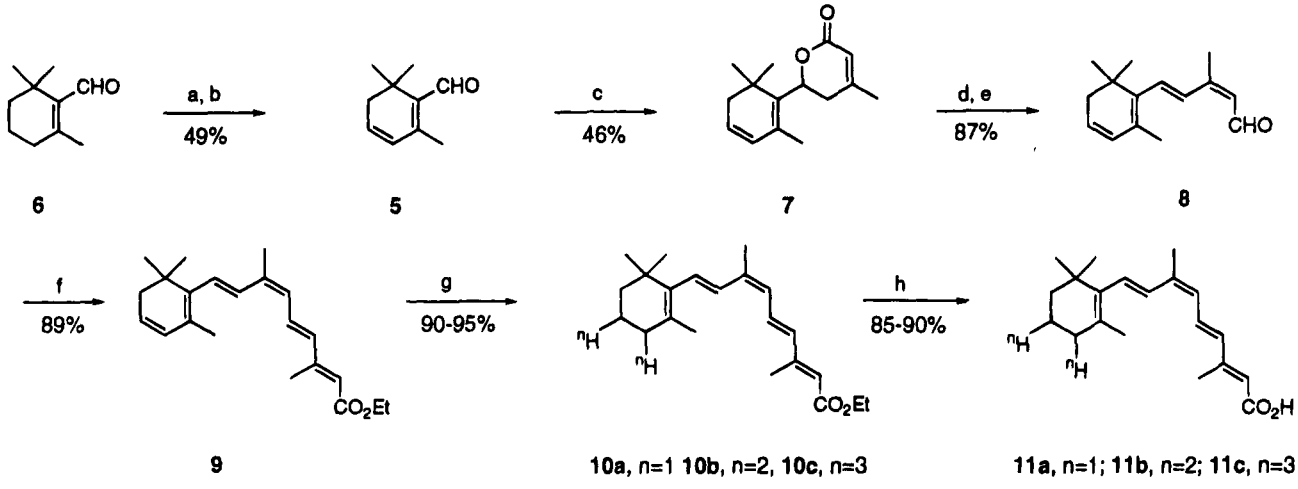


Figure 2.

Scheme 1



[a] Key: (a) NBS, CH_2Cl_2; (b) collidine, Δ; (c) LDA, ethyl dimethylacrylate; (d) DIBAL, THF; (e) HCl, $ClCH_2CH_2Cl$; (f) $(EtO)_2P(O)CH_2C(CH_3)=CHCO_2Et$, DMPU, THF, *n*-BuLi; (g) $Rh(PPh_3)_3Cl$, C_6H_6, $^nH_2$; (h) NaOH, EtOH.

Synthesis

The synthesis for ring-labeled 9-*cis*-RA presents several challenges which are as follows: (1) introduction of the 9-*cis* double bond and retention of its geometric integrity throughout the synthesis; (2) incorporation of the tritium labels near the final synthetic step, therefore decreasing the number of "radioactive synthetic steps" and increasing the radiochemical yield; and (3) devising an overall process that allows the preparation of high specific activity retinoids in an economical fashion.

We anticipated that tritiation of the endocyclic, disubstituted double bond in ethyl 3,4-didehydroretinoate **4** (Figure 2) would proceed in a regioselective fashion using Wilkinson's catalyst[11] and tritium gas. The radiolabeled ester **4** could then be hydrolyzed to the corresponding acid and purified via column chromatography or HPLC.

The preparation of 9-*cis*-ethyl retinoate **9** is outlined in Scheme 1. Allylic bromination of β-cyclocitral **6** with NBS in dichloromethane in the presence of calcium oxide and sodium bicarbonate at 0 °C, followed by elimination in boiling collidine, gave α-safranal **5** in 49% yield (two steps).[12] Treatment of aldehyde **5** with the lithium carbanion derived from ethyl 3,3-dimethylacrylate in THF at −78 to 0 °C afforded lactone **7** in 43−49% yields, along with the corresponding ω-hydroxy ester in similar yields.[13] The two products were easily separated by silica gel chromatography. DIBAL reduction of lactone **7** in THF at −78 °C to the corresponding lactol, followed by biphasic hydrochloric acid (1,2-dichloroethane: 10% HCl, 1:1) catalyzed ring opening, afforded the tetraenic aldehyde **8** in 87% yield for the two steps.[14] Horner−Wittig olefination with the appropriate phosphonate in the presence of DMPU at −78 to 0 °C gave ester **9** (89% yield) in a ratio of 13-*trans*:13-*cis* of ~15:1 (as determined by ^1H NMR).[15] Treatment of ethyl ester **9** in rigorously anhydrous oxygen-free benzene with $^nH_2$ (n = 1−3) in the presence of a stoichiometric amount of $(PPh_3)_3RhCl$ at 25 °C afforded the corresponding reduced materials **10a**−**10c** in greater than 90% yields. Several other catalysts, such as PtO_2, Pd/C, or Pt/C in a variety of solvents, were attempted but were all much less selective than Wilkinson's catalyst for the remote olefinic reduction. The esters **10a,b,c** were cleanly saponified to the corresponding acids **11a,b,c**. 3,4-$[^3H]_2$-9-*cis*-RA **11c** was purified by ODS-HPLC and characterized by ^1H, and ^3H NMR spectroscopy (specific activity 58 Ci/mmol).

The above methodology was extended to the preparation of 2,3-$[^3H]_2$-9-*cis*-RA (Scheme 2). Thus, in an analogous manner, ethyl 2,3-$[^3H]_2$-9-*cis*-RA ester **19** was prepared from 1,4-cyclohexadiene-1-carboxaldehyde **16**, as described below and subjected to the same tritiation method (*vide supra*).

Ethyl acetoacetate **12** (Scheme 2) was condensed with acetone in the presence of acetic anhydride and fused zinc chloride to afford 2-isopropylidene ketoester **13** in 38% yield.[16] Ketoester **13** was treated with allyltriphenylphos-

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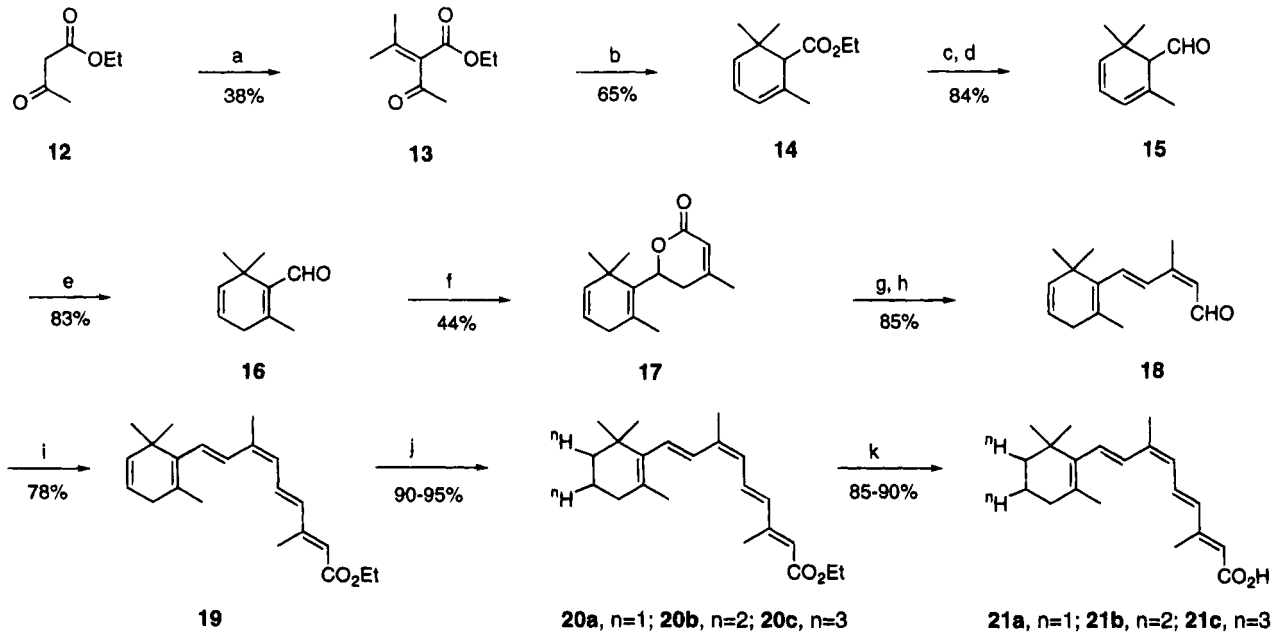
(15) Ratios vary between 10:1 and 15:1.

Scheme 2



[a] Key: (a) Acetone, Ac_2O, $ZnCl_2$; (b) allyltriphenylphosphonium chloride, *n*-BuLi, Et_2O; (c) $LiAlH_4$, Et_2O; (d) DMSO, $(COCl)_2$, Et_3N, THF; (e) DBU, CH_2Cl_2; (f) LDA, ethyl dimethylacrylate; (g) DIBAL, THF; (h) HCl, $ClCH_2CH_2Cl$; (i) $(EtO)_2P(O)CH_2C(CH_3)=CHCO_2Et$, DMPU, THF, *n*-BuLI; (j) $Rh(PPh_3)_3Cl$, C_6H_6, $^nH_2$; (k) NaOH, EtOH.

phoranyl ylide in a Michael–Wittig fashion to give cyclic ester **14** in 65% yield, according to the method of Wuest and Büchi.[17] $LiAlH_4$ reduction of ester **14** in diethyl ether, followed by Swern oxidation,[18] gave aldehyde **15** in 84% yield for the two steps. Isomerization of aldehyde **15** to **16** was accomplished cleanly using a catalytic amount (10%) of DBU in dichloromethane at room temperature in 83% yield. Treatment of aldehyde **16**, under the same conditions as described above for aldehyde **5**, gave lactone **17** in 44% yield, which was further reduced with DIBAL in THF at −78 °C to the corresponding lactol. Acid-catalyzed ring opening of the lactol intermediate gave tetraene aldehyde **18** in 87% yield. Chain extension to 2,3-didehydroretinoate **19** was achieved through an olefination reaction as above in 78% yield.

Treatment of ester **19** in anhydrous oxygen-free benzene with $^nH_2$ (n = 1–3) in the presence of a stoichiometric amount of $Rh(Cl)(PPh_3)_3$ afforded the corresponding reduced ester **20** in 85–90% yield. The 2,3-[^{3}H]$_2$-9-*cis*-RA **21c** was purified and characterized in the same manner as for **11c** and was shown to be labeled (95%) predominantly in the 2,3-positions, along with ~5% labeling at the 4-position (specific activity of 60 Ci/mmol). The compounds were purified by ODS-HPLC.

Discussion and Conclusions

3,4-[$^3H_2$]-9-*cis*-retinoic acid proved to be useful for competitive binding studies. However, this material could not be used for metabolic studies due to its high propency for oxidation to the corresponding 4-oxo retinoic acid, thus allowing for partial loss of the radiolabel in biological fluids. For these reasons, we turned our attention to the synthesis of 2,3-[$^3H_2$]-9-*cis*-retinoic acid using a similar labeling methodology.

A major concern during the regioselective reduction of ester **19** was the possible isomerization of the endocyclic 2,3-double bond (1,4-cyclohexadienyl system) to the more thermodynamically stable conjugated 1,3-cyclohexadienyl system **9** prior to reduction. Although under oxygen or ethanol-free benzene conditions double bond isomerization is rare using Wilkinson's catalyst,[11] we were concerned about the integrity of the 1,4-cyclohexadiene system in **19**. Isomerization of the remote olefin in compound **19** prior to reduction would give rise to the undesired 3,4-labeled compound **11**. Early results were encouraging when deuterium was used under the same conditions (*vide supra*) and showed a high degree of selectivity for the 2,3-double bond (^{1}H NMR). Fortunately, when the experiment was conducted with tritium, comparative NMR data analysis, **10b**, **21b** (^{1}H NMR) and **10c**, **21c** (^{3}H NMR), showed that less than 5% isomerization occurred during this reduction. ^{3}H-NMR data indicate that a small amount of tritium is indeed located at the 4-position of the ring, presumably due to reduction of the 3,4-olefin which resulted from rhodium catalyzed isomerization of the 1,4-cyclohexadienyl system in **19**.

In summary, two routes have been described to synthesize novel and high specific activity 2,3- and 3,4-ditritio-9-*cis*-retinoic acids. The main advantages of these methods are as follows: (1) the introduction of the tritium label at the penultimate step, which reduces the number of radioactive synthetic steps, (2) incorporation of high levels of radioactivity into this material, and (3) lower susceptibility of the ring-labeled compounds to loss of the radiolabel through oxidative degradation of the olefinic chain. These methods were utilized to prepare high specific activity and radiochemically pure 2,3-[$^3H_2$]-9-*cis*-retinoic acid which is extensively used for competitive binding and metabolic studies. In addition, the synthetic methodology utilized for this synthesis is versatile and may be applicable to the tritium and deuterium labeling of other retinoic acid analogues and synthetic retinoids as well as many natural products.

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Experimental Section

All the reactions were carried out under a nitrogen atmosphere except where stated. The organic solvents were purchased from Fisher Scientific, THF was distilled from Na (metal) in the presence of benzophenone, and diethyl ether was distilled from CaH_2. Thin layer chromatography was performed on Merck Kieselgel 60 F-254 plates. Reactions were monitored using both UV and an aqueous solution of ammonium molybdate and cerium sulfate for staining.[19] HPLC was performed on a Beckman C18 Ultrasphere (5 mm, 10 mm × 25 cm) column. Scintillation counting used Ecoscint A scintillation solution (National Diagnostics). SGC refers to silica gel chromatography. Compounds **5**,[13a] **8**, and **9** were previously reported in the literature[13c] and were prepared with minor modifications.

α-Safranal (5). A solution of β-cyclocitral (50.0 g, 0.328 mol) in CH_2Cl_2 (100 mL) was added to a suspension of $NaHCO_3$ (50.0 g, 0.59 mol) and CaO (12.5 g, 0.223 mol) in CH_2Cl_2 (100 mL), and the mixture was cooled to ~−7 °C (ice−acetone bath). *N*-Bromosuccinimide (59.04 g, 0.328 mol) was added in four portions over a 60 min period while the reaction temperature was maintained below 10 °C for 2 h with vigorous stirring. The cold reaction mixture was filtered and rinsed with cold CH_2Cl_2 (50 mL), and the filtrate was evaporated to provide a residue which was taken up in a 4:1 hexanes:ethyl acetate mixture (100 mL) and filtered. The filtrate was evaporated to give a yellow residue. (GC analysis showed 89% conversion to 4-bromo-β-cyclocitral containing 5.8% of α-cyclocitral; 94% adjusted yield.) The crude material was used directly in the next step. A 1-L three-neck, round-bottommed flask was charged with collidine (350 mL) and heated at 175 °C (oil bath temperature) under a nitrogen atmosphere, while the above crude 4-bromo-β-cyclocitral was added neat. The mixture was heated for 60 min and analyzed by GC (72% product and 28% byproducts). The mixture was cooled to room temperature and added to 300 mL of ice. Ether (400 mL) was added, followed by 6 N HCl (300 mL). The two phases were separated, and the organic layer was washed with 6 N HCl (200 mL), water (200 mL), satutated $NaHCO_3$ (200 mL), water (200 mL), and brine (200 mL). The washing sequence was repeated once, the organic layer dried over $MgSO_4$, and the solvent evaporated to give a dark oily material. Silica gel chromatography using a gradient from $CHCl_3$:hexanes (1:9 to 7:3) gave 23.5 g of 94% pure (GC) α-safranal, 49% yield. ^1H NMR ($CDCl_3$; 400 MHz) δ (ppm): 10.12 (s, 1 H), 6.15 (m, 1 H), 5.90 (m, 1 H), 2.1 (m, 5 H), 1.18 (s, 6 H).

5,6-Dihydro-4-methyl-6-(2,6,6-trimethylcyclohexa-1,3-dien-1-yl) -2*H*-pyran-2-one (7). A solution of lithium diisopropylamide was prepared as follows: A flame-dried 200-mL round-bottom flask was charged with anhydrous THF (100 mL) and anhydrous diisopropylamine (distilled over CaH_2 and kept over KOH under a nitrogen atmosphere) (3.20 mL, 23.0 mmol) and cooled to −20 °C. A solution of *n*-BuLi in hexanes (9.35 mL of a 2.35 M solution; 22.0 mmol) was added over a 10 min period. The solution was stirred at −20 °C for 15 min and then cooled to −78 °C. A solution of ethyl dimethylacrylate (Aldrich, Inc.) (2.69 g, 21.0 mmol) in THF (5.0 mL) was added slowly at such a rate that the internal temperature did not exceed −70 °C. The mixture was stirred at −78 °C for 30 min, after which time a solution of α-safranal (3.0 g, 20.0 mmol) in THF (25 mL) precooled at −78 °C was added dropwise. When the addition was complete, the reaction mixture was allowed to warm to 0 °C and then quenched with a saturated sodium bicarbonate solution (3.0 mL). The mixture was stirred at room temperature for 60 min, water (10 mL) was added, and the mixture was extracted with EtOAc (2 × 50 mL). The organic layer was washed with brine (2 × 20 mL), dried over $MgSO_4$, and concentrated. The residue was purified by SGC using a mixture of hexanes:ethyl acetate 5:1 as eluent to give 2.13 g of the desired lactone, 46% yield as an oil. IR ν_{max} cm^{-1} (neat): 2958 (CH aliph), 1720 (C=O), 1383 (C=C), 1294, 1251. ^1H NMR ($CDCl_3$; 400 MHz) δ (ppm): single diastereomer 5.82 (s, 1 H, CHC(O)O−), 5.75 (m, 2 H), 5.21 (dd, J = 13.3, 4.4 Hz, 1 H) 2.84 (m, 1H), 2.15 (m, 2 H), 2.12 (dd, J = 16.3, 4.4 Hz), 1.97 (s, 3 H), 1.81 (s, 3 H), 1.08 (s, 3 H), 1.04 (s, 3 H). ^{13}C NMR ($CDCl_3$; 100 MHz) δ (ppm): 165.5, 157.7, 133.9, 129.9, 129.6, 125.9, 116.2, 75.7, 40.2, 34.7, 33.99, 26.7, 25.7, 22.7, 19.2. *m/z*: 232, 217, 111. HRMS: calcd for $C_{15}H_{20}O_2$ 232.1463, found 232.1440.

(2Z,4E)-3-Methyl-5-(2,6,6-trimethylcyclohexa-1,3-dien-1-yl)penta-2,4-dien-1-al (8). A solution of 5,6-dihydro-4-methyl-6-(2,6,6-trimethylcyclohexa-1,3-dien-1-yl)-2*H*-pyran-2-one (**7**) (427 mg, 1.84 mmol) in THF (15.0 mL) was cooled at −78 °C, and a solution of diisobutylaluminum hydride in toluene (2.0 mL of a 1.0 M solution) was slowly added. After 15 min, TLC (hexanes:EtOAc 4:1) showed the reaction was complete. A saturated solution of Rochelle salt (10.0 mL) was added at −78 °C, and the mixture was allowed to warm to room temperature. EtOAc (30 mL) was added, and the layers were separated. The aqueous layer was thoroughly extracted with EtOAc (3 × 20 mL). The combined organic layers were washed with brine (20 mL) and concentrated to give 422 mg of virtually pure desired lactol in 98% yield. The lactol, obtained as white solid, mp 146−148 °C, was directly used in the next step: A solution of 5,6-dihydro-4-methyl-6-(2,6,6-trimethylcyclohexa-1,3-dien-1-yl)-2*H*-pyran-2-ol (422 mg, 1.80 mmol) in 1,2-dichloroethane (5.0 mL) was added to 10% HCl (5.0 mL), and the biphasic system was heated at 55 °C with vigorous stirring. The reaction was monitored by TLC until completion. The mixture was cooled to room temperature and carefully neutralized using a $NaHCO_3$ saturated solution. The layers were separated, and the aqueous phase was extracted with dichloromethane (2 × 20 mL). The combined organic extracts were washed with water (2 × 5 mL) and brine (20 mL), dried over $MgSO_4$, and concentrated. The residue was purified on a short silica gel column to give 348 mg (87% yield) of the desired aldehyde as a yellow oil. IR ν_{max} cm^{-1} (neat): 2958, 2928, 2866, 1668 , 1614. ^1H NMR ($CDCl_3$; 400 MHz) δ (ppm): 10.21 (d, J = 8.0 Hz), 7.30 (d, J = 16.0 Hz), 6.40 (d, J = 16.0 Hz), 5.88 (d, J = 8.0 Hz, 1H), 5.58 (m, 1 H), 5.49 (m, 1 H), 2.40 (s, 2 H), 2.18 (s, 3 H), 1.90 (s, 3 H), 1.10 (s, 6 H).

Ethyl (2*E*,4*E*,6*Z*,8*E*)-3,7-Dimethyl-9-(2,6,6-trimethylcyclohexa-1,3-dien-1-yl)nonatetraenoate (9). A solution of diethyl 3-(ethoxycarbonyl)-2-methylprop-2-enylphosphonate (1.76 g, 6.66 mmol) in anhydrous THF (10.0 mL) was cooled to 0 °C and treated with anhydrous DMPU (1.65 mL) and *n*-BuLi in hexanes (2.81 mL of 2.5 M solution, 7.59 mmol). The mixture was stirred at this temperature for 20 min and then cooled to −78 °C. A solution of aldehye **8** (800 mg, 3.70 mmol) in THF (10.0 mL) was slowly added and the reaction mixture stirred at −78 °C for an additional 60 min. The mixture was allowed to warm to 0 °C to effect completion of the reaction (TLC). A saturated solution of ammonium chloride (15 mL) was added and the mixture extracted with EtOAc (3 × 20 mL). The organic layer was washed with water (2 × 10 mL) and brine (20 mL), dried over $MgSO_4$, and concentrated. The residue was purified on a short SGC column to give 1.08 g (89% yield) of the desired ester in a ~15:1 ratio of 13-*trans*:13-*cis* isomers. IR ν_{max} cm^{-1} (neat): 2928, 1709; ^1H NMR ($CDCl_3$; 400 MHz) δ (ppm) 7.12 (dd, J = 15, 11.3 Hz, 1 H), 6.68 (d, J = 16 Hz, 1 H), 6.29 (d, J = 15 Hz, 1 H), 6.23 (d, J = 15 Hz, 1 H), 6.06 (d, J = 11.3 Hz, 1 H), 5.9 (d, J = 9 Hz, 1 H), 5.8 (m, 2 H), 4.15 (q, J = 7 Hz, 2 H), 2.37 (s, 3 H), 2.10 (s, 2 H), 2.03 (s 3 H), 1.75 (s, 3 H) 1.27 (t, J = 7 Hz, 3 H), 1.02 (s, 6 H). HRMS: calcd for $C_{22}H_{30}O_2$ 326.2245, found 326.2238.

Ethyl (2*E*,4*E*,6*Z*,8*E*)-3,7-Dimethyl-9-(3,4-[^{3}H]-2,6,6-trimethylcyclohex-1-en-1-yl)nonatetraenoate (10c). A suspension of $(PPh_3)_3RhCl$ (Wilkinson's catalyst) (180 mg, 0.195 mmol) in 2 mL of anhydrous benzene was degassed under N_2−freeze−thaw conditions, followed by addition of tritium gas (at 750 mm Hg) at 25 °C. After being stirred for 2 h under a tritium atmosphere the mixture turned to a homogeneous yellow-orange color. To this stirring mixture was added 30 mg (0.092 mmol) of ester **9** in 1 mL of anhydrous degassed benzene. The solution was stirred for 2 h followed by addition of hexane (5 mL) and filtration through silica gel. The filtrate

(19) Kriehner, J. G. *Thin Layer Chromatography*; Interscience: New York, 1967; p 151.

was concentrated and the resulting crude product used directly in the next step.

(2*E*,4*E*,6*Z*,8*E*)-3,7-Dimethyl-9-(3,4-[^{3}H]$_2$-2,6,6-trimethylcyclohex-1-en-1-yl)nonatetraenoic Acid (11c). The crude product **10c** was dissolved in EtOH (5 mL), followed by addition of 2 mL of 5 N aqueous NaOH. The reaction was heated to reflux for 30 min and cooled to 0 °C, followed by acidification with 1 N aqueous HCl. The mixture was extracted with EtOAc (3 × 5 mL), and the combined extracts were washed with brine, dried over $MgSO_4$, and concentrated. HPLC purification (C_{18}-ODS, 65% CH_3CN–35% H_2O–0.5% AcOH) gave 3.2 Ci of pure [^{3}H]$_2$-9-*cis*-retinoic acid **11c**, specific activity 58 Ci/mmol (the specific activity of this material was calculated from an aliquot of known concentration (calculated from the UV absorbance of the ϵ value for 9-*cis*-retinoic acid) and total radioactivity of the aliquot (scintillation counting of 1 mL of the aliquot)). The compound was 99.7% radiochemically pure (by radiochemical detector) and 98.5% chemically pure (by $UV_{264\ nm}$ detector and HPLC). R_f (20% EtOAc-hexane) = 0.26. UV_{MeOH} = 343 nm. ^{1}H-NMR (300 MHz) (CD_3OD) δ (ppm): 7.11 (dd, J = 12, 16 Hz, 1 H), 6.70 (d, J =16 Hz, 1 H), 6.30 (d, J =16 Hz, 1 H), 6.24 (d, J =16 Hz, 1 H), 6.11 (d, J = 12 Hz, 1 H), 5.79 (s, 1 H), 2.29 (s, 3 H, CH_3), 2.04 (t, J = 6 Hz, 2 H, CH_2), 2.00 (s, 3 H), 1.75 (s, 3 H), 1.62 (m, 1 H), 1.48 (t, J = 6 Hz), 1.05 (s, 6 H); ^{3}H-NMR (CD_3OD) δ (ppm) 2.03 (d, J = 7 Hz), 1.64 (d, J = 7 Hz).

Ethyl 2,2,6-Trimethylcyclohexa-3,5-diene-1-carboxylate (14). A flame-dried 2-L, three-necked, round-bottom flask was charged, under a nitrogen atmosphere, with allyltriphenylphosphonium chloride (Aldrich, Inc.) (66.86 g, 0.197 mol) and anhydrous diethyl ether (800 mL). The suspension was cooled to 0 °C, and *n*-BuLi in hexanes (126.0 mL of a 1.6 M solution, 0.2 mol) was added dropwise over a 30 min period through an addition funnel. The orange mixture was stirred at 0 °C for 60 min followed by the addition of ethyl isopropylideneacetoacetate[16] **13** (30.5 g, 0.179 mol) in anhydrous diethyl ether (150 mL) over a 15 min period. The reaction mixture was stirred at room temperature for 60 min, and then cold water (100 mL) was added slowly. The two phases separated. The aqueous layer was extracted with diethyl ether (200 mL), and the combined organic extracts were successively washed with water (100 mL) and a saturated brine solution (2 × 50 mL). The organic phase was dried over $MgSO_4$ and concentrated *in vacuo* to give a semisolid reddish material which was passed over a short silica gel column (10 × 8 cm) using a mixture of hexanes:ethyl acetate 4:1. The appropriate fractions were combined and concentrated and the residue distilled at 93–95 °C at 8 Torr to give 22.68 g of pure ester **14** (48% yield). IR ν_{max} cm^{-1} (neat): 2964 1738, 1446. ^{1}H NMR ($CDCl_3$; 400 MHz) δ (ppm): 5.76 (m, 1 H, H_4), 5.74 (s, 1 H), 5.35 (d, J = 9.6 Hz, 1 H), 4.10 (q, J = 7.0 Hz, 2 H), 2.74 (s, 1 H), 1.75 (s, 3 H), 1.21 (t, J =7.0 Hz 3 H), 1.05 (s, 3 H), 1.03 (s, 3 H). ^{13}C NMR ($CDCl_3$; 100 MHz) δ (ppm): 171.9, 134.3, 130.8, 121.3, 120.3, 60.0, 56.7, 34.4, 28.1, 24.4, 22.3, 14.2. *m/z*: 194, 137, 121, 105, 91, 79. HRMS: calcd for $C_{12}H_{18}O_2$ 194.1307, found 194.1316.

2,6,6-Trimethylcyclohexa-2,4-diene-1-carboxaldehyde (15). A solution of ethyl 2,6,6-trimethyl-2,4-cyclohexadiene-1-carboxylate (**14**) (9.09 g, 0.0468 mol) in anhydrous diethyl ether (100 mL) was slowly added to a suspension of $LiAlH_4$ (3.75 g, 0.099 mol) in diethyl ether (200 mL) at 0 °C. The mixture was stirred for 4 h and monitored by thin layer chromatography (TLC). Water (4.0 mL) was carefully added, followed by 15% NaOH (4.0 mL) and water (12 mL). The white precipitate was filtered and rinsed with warm EtOAc (100 mL). EtOAc (100 mL) added, and the combined organic layers were washed with water (3 × 50 mL) and brine (3 × 50 mL), dried ($MgSO_4$), and concentrated. The residue was purified by SGC (hexanes:EtOAc 4:1) to give 6.15 g (87% yield) of the pure 2,6,6-trimethylcyclohexa-2,4-diene-1-methanol. IR ν_{max}cm^{-1} (neat): 3340, 2958, 1467, 1442, 1375, 1357, 1049, 1012. ^{1}H NMR ($CDCl_3$; 400 MHz) δ (ppm): 5.76 (m, 1 H), 5.69 (m, 1 H), 5.36 (d, 9.4 Hz, 1 H), 3.73 (m, 2 H), 1.85 (s, 3 H), 1.77 (t, J = 4.1 Hz, 1 H), 1.55 (t, J = 5.0 Hz, 1 H), 1.14 (s, 3 H), 1.02 (s, 3 H). ^{13}C NMR ($CDCl_3$; 100 MHz) δ (ppm): two diastereomers 136.6, 135.2, 135.0, 122.3, 120.7, 120.3, 118.8, 63.1, 61.7, 61.6, 60.3, 52.2, 50.9, 33.7, 28.1, 26.9, 25.4, 24.1, 22.5, 21.2. *m/z*: 152, 121, 105, 91, 79. HRMS: calcd for $C_{10}H_{16}O$ 152.1201, found 152.1197.

A flame-dried 500-mL round-bottom flask was charged, under a nitrogen atmosphere, with THF (150 mL) and oxalyl chloride (4.25 mL, 0.049 mol) and then cooled to −78 °C in an acetone–dry ice bath. Dimethyl sulfoxide (DMSO) (6.95 mL, 0.097 mol) was slowly added, and the mixture was warmed to −35 °C for 3 min and cooled again to −78 °C. A solution of 2,2,6-trimethylcyclohexa-3,5-diene-1-methanol (5.50 g, 0.0362 mol) in anhydrous THF (50 mL) was added dropwise. The mixture was stirred at −78 °C for an additional 20 min and warmed to −35 °C for 30 min. The turbid solution was cooled back to −78 °C and added with triethylamine (14.65 mL, 0.105 mol) and then allowed to warm to room temperature. A saturated solution of ammonium chloride (50 mL) was added, followed by EtOAc (100 mL). The organic layer was washed with water (3 × 50 mL) and brine (50 mL), dried over $MgSO_4$, and concentrated. The residue was purified by SGC using a mixture of hexanes:EtOAc 10:1 as eluent to give 5.31 g of the title compound **15** in 96% yield. IR ν_{max} cm^{-1} (neat): 2960, 1716. ^{1}H NMR ($CDCl_3$; 400 MHz) δ (ppm): 9.29 (d, J = 5.5 Hz, 1 H), 5.9 (m, 1 H), 5.75 (m, 1 H), 5.43 (d, J = 9.4 Hz, 1 H), 2.28 (d, J = 5.3 Hz, 1 H), 1.71 (s, 3 H), 1.06 (s, 3 H), 1.00 (s, 3 H). ^{13}C NMR ($CDCl_3$; 100 MHz) δ (ppm): two diastereomers 200.3, 134.6, 129.2, 122.3, 122.1, 63.9, 33.6, 31.8, 29.3, 26.4, 25.6, 22.6, 22.1. *m/z*: 150, 121, 105, 91, 81. HRMS: calcd for $C_{10}H_{14}O$ 150.1045, found 150.1048.

2,6,6-Trimethylcyclohexa-1,4-diene-1-carboxaldehyde (16). To a stirred solution of 2,2,6-trimethylcyclohexa-3,5-diene-1-carboxaldehyde (**15**) (3.70 g, 24.8 mmol) in anhydrous dichloromethane (25 mL) at 0 °C was added 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (0.150 mL, 0.98 mmol) in three portions over 30 min. The solution was warmed to room temperature and the reaction monitored by TLC (hexanes:EtOAc 9:1). The two isomeric aldehydes were easily separated on TLC (R_f of **15**: 0.85; R_f product **16**: 0.75). After 60 min, the reaction did not proceed further. A saturated solution of ammonium chloride (20 mL) was added followed by dichloromethane 100 mL. The organic layer was separated and washed with a saturated solution of brine, dried over $MgSO_4$, and concentrated. The residue was purified by SGC using a mixture of hexanes:EtOAc 15:1 as eluent to give 3.1 g of the desired aldehyde in 83% yield. IR ν_{max} cm^{-1} (neat): 2960 CH), 1716 (C=O). ^{1}H NMR ($CDCl_3$; 400 MHz) δ (ppm): 10.18 (s, 1 H), 5.44, (m, 2 H), 2.81 (s, 2 H, $-CH_2-$), 2.12 (s, 3 H), 1.25 (s, 6 H). ^{13}C NMR ($CDCl_3$; 100 MHz) δ (ppm): 191.8, 152.1, 138.4, 117.4, 35.6, 34.2, 28.1, 22.6, 18.47, 14.0. *m/z*: 150, 135, 121, 107, 91, 77. HRMS: calcd for $C_{10}H_{14}O$ 150.1045, found 150.1052.

5,6-Dihydro-4-methyl-6-(2,6,6-trimethylcyclohexa-1,4-dien-1-yl)-2*H*-pyran-2-one (17). This lactone was prepared from aldehyde **16** in 44% yield according to the procedure described for **7**. Mp: 77–79 °C. IR ν_{max} cm^{-1} (neat): 2958, 1720, 1383, 1294, 1251. ^{1}H NMR ($CDCl_3$; 400 MHz) δ (ppm): 5.82 (s, 1 H), 5.50 (m, 1 H), 5.39 (m, 1 H), 5.01 (dd, J = 13.3, 4.4 Hz, 1 H) 2.84 (m, 1H), 2.60 (m, 2 H), 2.12 (dd, J = 16.3, 4.4 Hz, 1 H), 1.97 (s, 3 H), 1.79 (s, 3 H)1.13 (s, 3 H, CH_3 ring), 1.01 (s, 3 H). ^{13}C NMR ($CDCl_3$; 100 MHz) δ (ppm): 165.8, 157.6, 135.8, 132.5, 131.6, 119.9, 116.2, 75.3, 35.9, 35.2, 34.18, 28.9, 28.5, 22.8, 20.5. *m/z*: 232, 217, 111. HRMS: calcd for $C_{15}H_{20}O_2$ 232.1463, found 232.1445.

(2*Z*,4*E*)-3-Methyl-5-(2,6,6-trimethylcyclohex-1,4-dien-1-yl)penta-2,4-diene-1-al (18). This aldehyde was prepared in two steps by (1) reduction of lactone **16** to 5,6-dihydro-4-methyl-6-(2,6,6-trimethylcyclohexa-1,4-dien-1-yl)-2*H*-pyran-2-ol (**17**) in 98% yield, mp 146–148 °C, as a single diastereomer (however, both in chloroform solution or upon sgc this compound isomerized to an anomeric mixture (~55:45)) [IR ν_{max} cm^{-1} (KBr): 3387, 2935, 1688 (weak). ^{1}H NMR ($CDCl_3$; 400 MHz) δ (ppm): two distereomers 5.52 (m, 2 × 2 H), 5.39 (s 2 × 1 H), 5.35 (m, 2 × 1 H), 4.71 (dd, J = 11.2, 4.4 Hz, 1 H), 4.3 (dd, J = 13, 4.4 Hz, 1 H), 3.0 (d, J = 11 Hz, 1 H), 2.88 (d, J = 4.2 Hz, 1 H) 2.7–2.45 (m 2 × 4 H), 1.87 (s 3 H), 1.81 (s, 3 H), 1.73, (s, 3 H), 1.70 (s, 3 H), 1.13 (s, 3 H), 1.1 (s, 3 H), 1.01 (s, 3 H), 0.99 (s 3 H). ^{13}C NMR ($CDCl_3$; 100 MHz) δ (ppm): major

137.8, 136.2, 134.9, 129.9, 119.9, 119.8, 89.7, 65.2, 36.4, 35.4, 34.1, 28.8, 28.4, 22.7, 20.2; minor 137.6, 135.9, 134.5, 129.8, 122.7, 116.3, 93.0, 70.73, 35.9, 35.4, 34.0, 28.8, 28.4, 22.5, 19.8. *m/z*: 232, 217, 111. HRMS: calcd for $C_{15}H_{20}O_2$ 234.1620, found 234.1625] and (2) ring opening of **17** to aldehyde **18**. IR ν_{max} cm^{-1} (neat): 2958, 2928, 2866, 1668 (C=O), 1614 (C=C). ^1H NMR ($CDCl_3$; 400 MHz) δ (ppm): 10.16 (d, J = 8.0 Hz), 7.16 (d , J = 16.0 Hz), 6.63 (d , J = 16.0 Hz), 5.88 (d, J = 8.0 Hz, 1H), 5.58 (m, 1 H), 5.49 (m, 1 H), 2.69 (s, 2 H), 2.14 (s, 3 H), 1.80 (s, 3 H), 1.11 (s, 6 H). ^{13}C NMR ($CDCl_3$; 100 MHz) δ (ppm): 201.0, 190.0, 154.8, 136.6, 135.6, 135.5, 129.3, 128.3, 128.0, 120.1, 35.7, 33.5, 29.5, 21.1, 21.0. HRMS: calcd for $C_{15}H_{20}O$ 218.1670, found 218.1667. Both steps were performed according to the procedures described above for aldehyde **8**.

Ethyl (2*E*,4*E*,6*Z*,8*E*)-3,7-Dimethyl-9-(2,6,6-trimethylcyclohexa-1,4-dien-1-yl) nonatetraenoate (19). This compound was prepared in 78% yield following the procedure described above for ester **9**. IR ν_{max} cm^{-1} (neat): 2928, 1709. ^1H NMR ($CDCl_3$; 400 MHz) δ (ppm): 7.08 (dd, J = 15, 11.3 Hz, 1 H), 6.75 (d, J = 16 Hz, 1 H), 6.29 (d, J = 15 Hz, 1 H), 6.23 (d, J = 15 Hz, 1 H), 6.06 (d, J = 11.3 Hz, 1 H), 5.9 (s, 1H), 5.6 (m, 1 H), 5.52 (m, 1 H), 4.17 (q, J = 7 Hz, 2 H), 2.7 (s, 2 H), 2.33 (s, 3 H), 2.03 (s 3 H), 1.82 (s, 3 H) 1.29 (t, J = 7 Hz, 3 H), 1.1 (s, 6 H). HRMS: calcd for $C_{22}H_{30}O_2$ 326.2245, found 326.2240.

Ethyl (2*E*,4*E*,6*Z*,8*E*)-3,7-Dimethyl-9-(2,3-[^{3}H]$_2$-2,6,6-trimethylcyclohex-1-en-1-yl)nonatetraenoate (20c). This compound was prepared from ester **19** following the procedure described for **10c**.

(2*E*,4*E*,6*Z*,8*E*)-3,7-Dimethyl-9-(2,3-[^{3}H]$_2$-2,6,6-trimethylcyclohex-1-en-1-yl)nonatetraenoic Acid (21c). This compound was prepared from ester **20c** according to the procedure described for **11c** and was 99.5% radiochemically pure (by radiochemical detector) and 98.7% chemically pure (by $UV_{264\ nm}$ detector and HPLC). R_f (20% EtOAc–hexane) = 0.26. UV_{MeOH} = 343 nm. ^1H-NMR (CD_3OD) δ (ppm): 7.11 (dd, J = 12, 16 Hz, 1 H), 6.70 (d, J =16 Hz, 1 H), 6.30 (d, J =16 Hz, 1 H), 6.0 (d, J =16 Hz, 1 H),6.11 (d, J = 12 Hz, 1 H), 5.79 (s, 1 H), 2.29 (s, 3 H), 2.03 (t, J = 6 Hz, 2 H), 2.0 (s, 3 H), 1.75 (s, 3 H), 1.63 (m, 1 H), 1.48 (t, J = 6 Hz), 1.05 (s, 6 H). ^{3}H-NMR (300 MHz) (CD_3OD) δ (ppm): 2.03 (d, J = 6 Hz, 1/20 ^{3}H), 1.48 (d, J = 6 Hz), 1.64 (d, J = 3 Hz).

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