THE SYNTHESIS OF ISOTOPICALLY LABELED RETINOIDS

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SUMMARY

Retinyl acetate (4a) has been prepared with a tritium label at the C-11 and C-12 positions by partial reduction of oxenin (1) with tritium gas followed by acetylation and rearrangement. Specific activities of up to 40 curies/mmole have been attained. By alkaline hydrolysis, retinol (5) has been obtained and derivatized as one of several retinyl esters or has been oxidized to all-trans retinoic acid (6) having equally high specific tritium activities. From beta ionone (12), 13-cis retinoic acid (10) has been elaborated in a variety of isotopically labeled forms. A key reaction in the sequence, the Wittig coupling of the triphenylphosphonium derivative of vinyl beta ionol (15a-c) and the butenolide, 5-hydroxy-4-methyl-2(5H)-furanone (8), provides access to 13-cis retinoic acid and its 4-oxo metabolite labeled with either isotopic hydrogen or carbon.

Key words: Oxenin, Wittig coupling, Labeled retinoids.

In preliminary communications, we reported[1,2] the preparation of retinol, retinyl acetate and all-trans retinoic acid nominally labeled with tritium at the 11 and 12 positions with specific activities ranging from 10 to 40 curies/mmole. The preparative details of these isotopically labeled compounds are now presented along with those of related retinoids which have not been previously reported.

Oxenin (1, Scheme 1) was disclosed by Isler, et al.,[3] over 40 years ago as a useful intermediate in the synthesis of vitamin A. Oxenin contains the requisite 20 carbon atoms of the vitamin A skeleton and partial reduction with hydrogen under Lindlar conditions provided hydroxenin (2). This reaction offers an excellent opportunity for labeling vitamin A and its derivatives with tritium and such labeling was indeed carried out[4] to provide products having relatively low specific activities. This procedure as well as other methods of preparing retinoids labeled to modest specific activities have been recently reviewed.[5]

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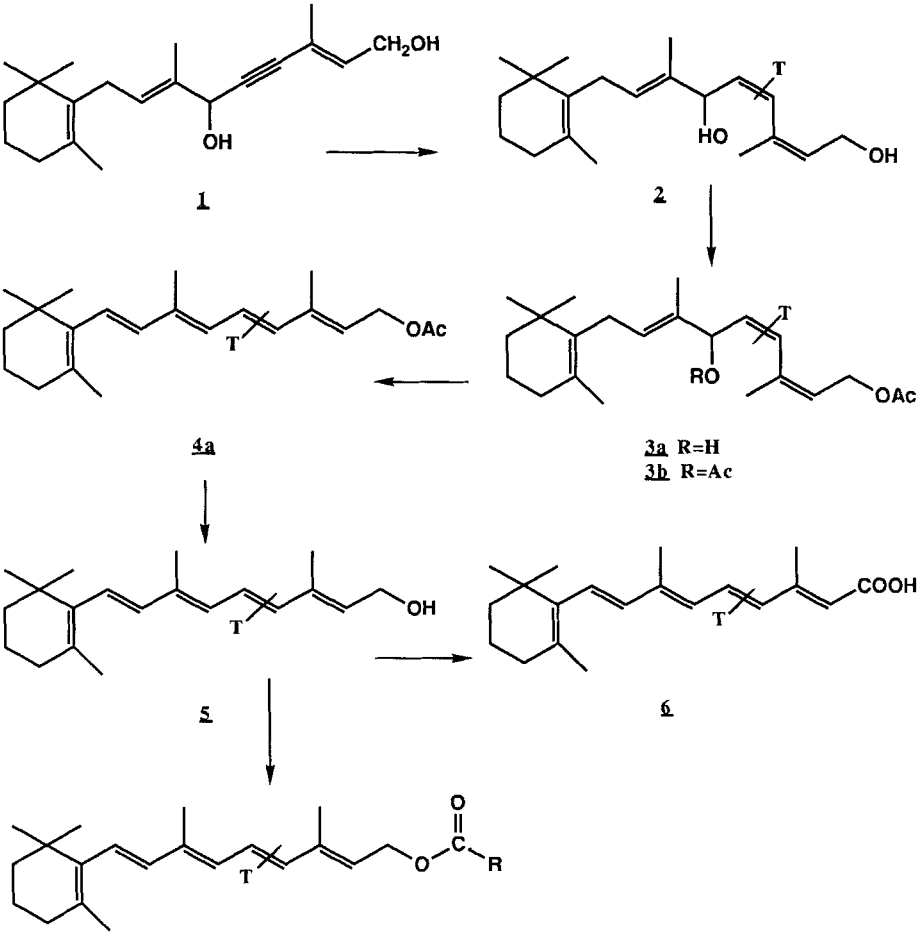
With the discovery and use of cellular retinoid binding proteins,[6] specific activities of tritium labeled retinoids in the 10-40 Ci/mmole range became necessary and the partial reduction of oxenin, now using carrier free tritium gas, is still the method of choice to obtain products having these high specific activities. On a 100 mg scale (0.33 mmole of oxenin), the use of 10 Ci of undiluted tritium gas (0.17 mmole) affords hydroxenin at specific activities up to 40-50 Ci/mmole after workup. This material can be carried on to the desired retinoid products having the same high specific activity but in quite modest chemical yield. However, if the hydroxenin reduction mixture, after removing any unreacted tritium gas, is treated with an atmosphere of unlabeled hydrogen, a considerably improved chemical yield of somewhat lower specific activity product is obtained. In either case, the hydroxenin that is formed is readily separated from starting material and from over-reduced product by chromatography over silica gel. Dilution of the purified hydroxenin-^{3}H with unlabeled hydroxenin to a total mass of about 50 mg facilitates the ensuing sequence and provides reasonable chemical yields of final product having specific activity in the 10-20 Ci/mmole range. As shown in Scheme 1, acetylation of 2 yields acetrene (3a), quantitatively, which undergoes rearrangement to retinyl acetate (4a) using 62% hydrobromic acid and the phase transfer reagent cetyl trimethylammonium bromide (CETAB). This rearrangement is quite sensitive to acidity, light, temperature and atmosphere but excellent yields of retinyl-11,12-$^3H_2$ acetate have been routinely obtained as described in the experimental section. The hydrolysis of 4a with sodium hydroxide in ethanol is straightforward and yields retinol (5) which is the most unstable of the tritium labeled retinoids. If stored as such, it rapidly undergoes radiolytic decomposition. If converted to retinoic-11,12-$^3H_2$ acid (6) by oxidation (via retinal) or to a retinyl ester (4b-d), significant radiochemical purity will be retained for periods of 4-6 months. In the oxidation process to produce 6, manganese dioxide and silver oxide are added directly to a methanolic sodium hydroxide solution of 4a and the resulting retinoic-11,12-$^3H_2$ acid is purified by crystallization to greater than 98% radiochemical purity. When purified and stored in toluene solution at a radiochemical concentration of about 1 mCi/mL and a total of up to 100 mCi at -60°C in light protected containers, significant decomposition of 6 does not take place for about 4 months. At that time, the material can be repurified by hplc.[5]

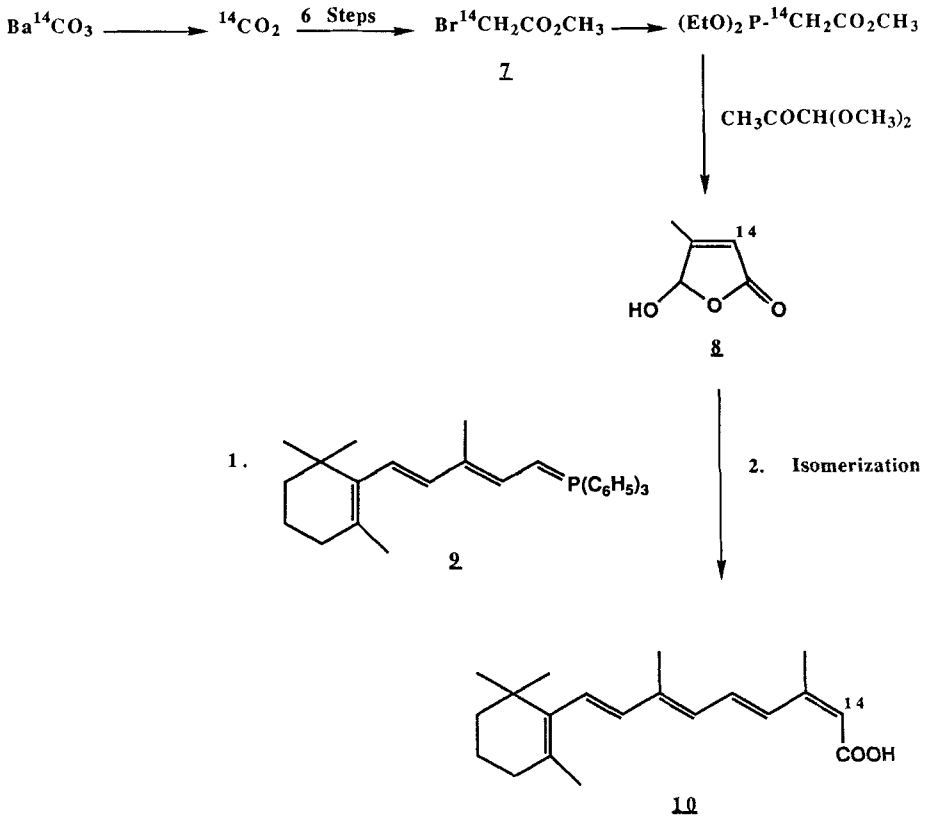
Retinyl esters that have been prepared include the propionate, myristate and palmitate and details of their syntheses are contained in the experimental section. Storage conditions for these esters are similar to those described above and their radiochemical stability is slightly better than that of 6.

Several years ago, we reported[7] the preparation of 13-cis retinoic acid labeled with carbon-14 in the 6 and 7 positions. At that time, the metabolism of 13-cis retinoic acid was unknown and labeling the molecule close to the cyclic structure was carried out with the assumption that these positions were more likely to be metabolically stable than those nearer the terminal carboxyl group. When the metabolism of 13-cis retinoic acid became

Scheme 1. Tritium Labeling from Oxenin



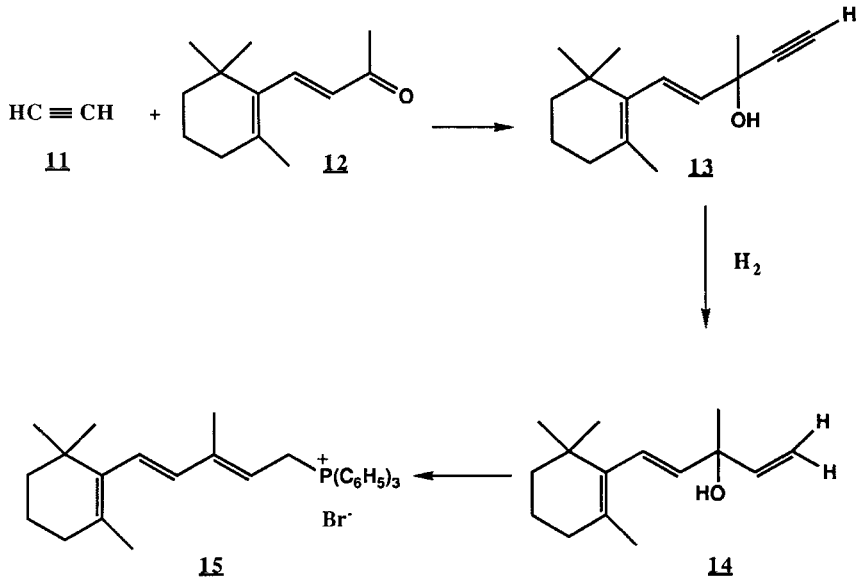
Scheme 2. Synthesis of 13-cis Retinoic-14-^{14}C Acid



known[8] and showed that the molecule remained largely intact, the chemistry reported for the preparation of the C-6,7 labeled material was then used to prepare other labeled forms but in fewer radiochemical steps. As shown in Scheme 2, the butenolide (8) that is conveniently used in the preparation of 13-cis retinoic acid,[9] was prepared in labeled form by the reaction of methyl bromoacetate-2-^{14}C (7) and triethyl phosphite followed by treatment with pyruvic aldehyde dimethylacetal. Upon reaction with the C-15 Wittig reagent (9), 13-cis retinoic acid (10) labeled with carbon-14 at the 14 position is obtained. Synthesis of labeled 8, which is radiochemically stable, provides a stock of intermediate which can be rapidly incorporated into the less stable product as it is needed.

The versatility of this approach to labeled retinoids is further enhanced in considering the various possibilities in labeling the Wittig reagent. Preparation of the phosphonium salt precursor to 9 is readily achieved as shown in Scheme 3. Reaction of acetylene with beta ionone (12) yields the alkynol 13 which undergoes partial hydrogenation to vinyl beta ionol (14) and then to the product (15) on reaction with triphenyl phosphine and hydrobromic acid. The phosphonium salt is relatively stable and has been stored for up to a year with a carbon-14 label that was prepared by using carbon-14 labeled acetylene in this synthesis.

Scheme 3. Synthesis of the C-15 Phosphonium Salt



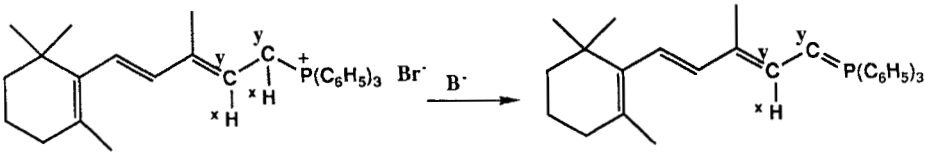
The partial reduction step, 13 ---> 14, can be carried out using either deuterium or tritium to produce the correspondingly labeled olefin 14 and the phosphonium salt 15. The tritium labeled phosphonium salt has been prepared and used in the synthesis of tritium labeled all-trans retinoic acid[10] as has the carbon-14 labeled phosphonium salt in the analogous synthesis of carbon-14 labeled all-trans retinoic acid.[5]

We have used this approach to prepare 13-cis retinoic acid that is labeled with deuterium or with tritium at C-10 or with carbon-14 at positions 10 and 11 as shown in Scheme 4. The Wittig reaction is carried out in greatest yield in a protic solvent and leads to the loss of the isotopic hydrogen atom at the 11 position (in the retinoic acid).[10] Since this is an equilibrium process, the loss occurs in converting the phosphonium salt (15a or 15b) to the corresponding ylid (9a or 9b). With carbon-14 labeling, the label is in the skeleton. No effect is seen and the label remains at both the 10 and 11 positions of the product (10c).

Stable isotope labeled standards of 13-cis retinoic acid and its 4-oxo metabolite have also been prepared by the use of this chemistry. Since mass spectral standards generally require an increase of 3 (or more) mass units over the parent compound, we prepared 13-cis retinoic acid labeled with 4 deuterium atoms as shown in Scheme 5. Beta ionone-$^2H_4$ (12a) was prepared from hexadeuteroacetone (17) and citral (16) via pseudo ionone (18).

The base catalyzed formation of 18 was carried out in methanol-OD with sodium hydroxide-OD and only the expected chemical loss of two atoms of deuterium occurred in this process. No significant exchange took place in the subsequent formation of beta ionone-$^2H_4$ and in its several step conversion to 13-cis retinoic-$^2H_4$ acid (10d) which had overall d_4 content of greater than 95%.

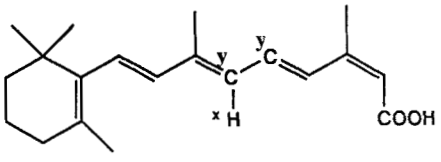
Scheme 4. Syntheses of Labeled Forms of 13-cis Retinoic Acid



15a x=2, y=12
15b x=3, y=12
15c x=1, y=14

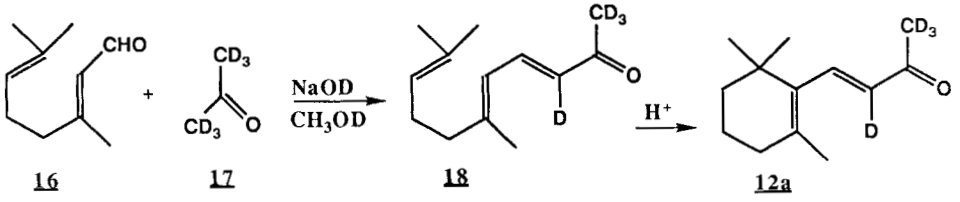
9a x=2, y=12
9b x=3, y=12
9c x=1, y=14

8, unlabeled

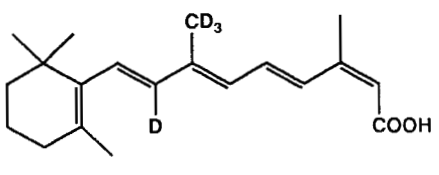


10a x=2, y=12
10b x=3, y=12
10c x=1, y=14

Scheme 5. Synthesis of 13-cis Retinoic-$^2H_4$ Acid



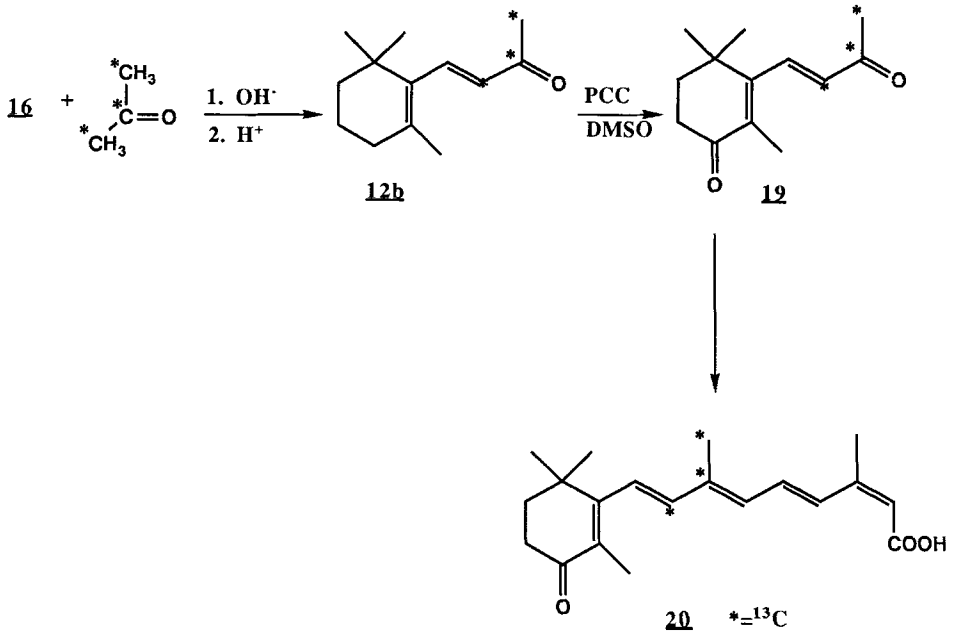
16 17 18 12a



10d

The main metabolite of 13-cis retinoic acid in mammals is the 4-oxo derivative (20, Scheme 6). Preparation of this compound can be achieved by pyridinium chlorochromate oxidation of beta ionone[11] to 4-oxo beta ionone which follows the course of reactions to 13-cis retinoic acid that are discussed above.[15] Accordingly, the oxidation was applied to beta ionone-$^{2}H_4$ but extensive exchange occurred and provided product with only about 50% d_4 content. A suitable standard was prepared from beta ionone-$^{13}C_3$ which had been prepared via the condensation of acetone-1,2,3-$^{13}C_3$ and citral (Scheme 6) in a sequence similar to that shown in Scheme 5. The carbon-13 labeled beta ionone was then oxidized to 4-oxo beta ionone-$^{13}C_3$ (19) and the rest of the sequence to 4-oxo-13-cis retinoic-$^{13}C_3$ acid (20)

Scheme 6. Synthesis of 4-oxo-13-cis Retinoic-$^{13}C_3$ Acid



was carried out exactly as described above for the parent compound. The presence of the cyclic ketone in 19 did not interfere in the subsequent reactions.

The retinoids that were prepared in isotopically labeled forms are listed in the table.

Table. Isotopically Labeled Retinoids

Compound	No. in Text	Isotope	Position of Label	Specific Activity/Enrichment
All-trans Retinoids				
Retinol	5	tritium	11,12	10 - 40 Ci/mmole
Retinyl acetate	4a	tritium	11,12	10 - 40 Ci/mmole
Retinyl propionate	4b	tritium	11,12	24.49 Ci/mmole
Retinyl myristate	4c	tritium	11,12	16 Ci/mmole
Retinyl palmitate	4d	tritium	11,12	16 Ci/mmole
Retinoic acid	6	tritium	11,12	10 - 40 Ci/mmole
13-cis Retinoids				
13-cis-Retinoic acid	10a	deuterium	10	100% (d_1)
13-cis-Retinoic acid	10d	deuterium	8, (9,9,9-methyl)	94% (d_4)
13-cis-Retinoic acid	10b	tritium	10	4.86 Ci/mmole
13-cis-Retinoic acid	10	carbon-14	14	5.37 mCi/mmole
13-cis-Retinoic acid	10c	carbon-14	10,11	27.9 mCi/mmole
4-keto-13-cis-Retinoic acid	20	carbon-13	8,9,19	99% ($^{13}C_3$)

EXPERIMENTAL

General. All solvents were distilled (tetrahydrofuran was distilled from sodium ribbon using benzophenone as indicator). Spectra were recorded on standard instruments by the staff of the Physical Chemistry Department of Hoffmann-La Roche Inc. Radiochemical purity was determined on thin layer chromatograms with an LB2832 Berthold Linear Analyzer System and radioactivity was measured by the liquid scintillation technique using either a Packard Tricarb Model 2010 or Beckman Model LS7500 spectrometer. Reactions to prepare the various retinoids were carried out under subdued lighting or under yellow lights.

Hydroxenin-11,12-$^3H_2$(2). "Reduction solvent" is prepared by dissolving 2 mg of "EDS" (3,6-dithia-1,8-octanediol) and 110 mg of dimethylethanolamine in 500 mL of heptane. In a 5 mL reaction flask equipped with a high-vacuum stopcock, 60 mg (0.2 mmole) of oxenin (1), 20 mg of regular Lindlar catalyst, and 3 mL of reduction solvent were combined, degassed, and reduced with 4cc (0.17 mmole, 10 Ci) of tritium gas for 6-7 hours at room temperature. Any remaining tritium was then pumped back into an ampule for storage, and the reaction mixture then filtered through a pad of Celite packed in a Pasteur pipette. The filtrate was then concentrated in vacuo to a near-theoretical weight of 59 mg, and stored overnight in 25 mL of argon-purged toluene containing 60 µg of BHA (added as a 100 ppm solution in toluene) at -15°. The mixture was then concentrated to dryness, at 30°/10 mm, and chromatographed over 30 g of silica gel (E. Merck #7734) packed and eluted with hexane 75, ethyl acetate 15, acetone 10 and pyridine, 0.5. Fractions of 6 mL were collected, with 2 drops of 100 ppm BHA/toluene in each tube. Fractions #22-27 were combined and concentrated in vacuo to a residual weight of 26.5 mg (44%) which by thin layer chromatography (tlc) on silica gel and elution with the solvent mixture described above, had an Rf value of 0.25 and radiochemical purity of 90%.

Acetrene-11,12-$^3H_2$ (3a). The hydroxenin-11,12-$^3H_2$, obtained above, was diluted to 50 mg with non-radioactive hydroxenin and the total dissolved in 5 mL of toluene. A solution of 137 mg of triethylamine and 141 mg of acetic anhydride in 2 mL of toluene was added and the resulting mixture was heated at 40°, under argon, in a closed flask for 18 hr. The mixture was chilled to 0°, 2 mL of 1M sodium carbonate was added and then stirred at 0°, under argon, for 2 hr. After this time, the phases were separated and the organic phase was extracted with two 2 mL portions of water. The aqueous phases were combined and extracted with 2 mL of toluene. The combined toluene phase was washed with 1 mL of brine, dried over anhydrous magnesium sulfate, filtered and concentrated in vacuo to a 56 mg residue containing about 80% of 3a and 20% of 3b as determined by TLC.

Retinyl-11,12-$^3H_2$ acetate (4a). "Rearrangement solvent" is prepared by dissolving 10 mg of cetyl trimethylammonium bromide (CETAB) and 10 µL of pyridine in 100 mL of methylene chloride. The crude acetrene-11,12-$^3H_2$, obtained above, was dissolved in 1.65 mL of "rearrangement solvent". The resulting solution, under argon, was chilled, with

stirring, to -47°, then 28.8 μL of cold 62% hydrobromic acid was added and the resulting mixture was stirred vigorously, at that temperature, for 1 minute. While maintaining stirring, the -47° bath was removed, 1.65 mL of 1M sodium carbonate solution was added and an ice-bath was placed around the reaction flask. At this temperature, the mixture was stirred rapidly for 3 hr, then diluted with 8.25 mL of water and 8.25 mL of methylene chloride. After phase separation, the aqueous phase was extracted with 8.25 mL of methylene chloride and the combined organic phases were then washed with 3.3 mL of water containing 3 drops of brine, dried over anhydrous granular potassium carbonate and filtered. BHA, 82.5 μg, and pyridine, 82.5 μL were added as 100 ppm toluene solutions and the mixture was concentrated in vacuo to a residue of 52 mg. This material was chromatographed over 39 g of silica gel (E. Merck #7734) packed in and eluted with hexane 90, anhydrous ether 10, pyridine 0.1. Fractions of 6 mL were collected; 2 drops of BHA solution (100 ppm/toluene) were placed in each tube before collection. Fractions 22-30 were combined and concentrated in vacuo to a residue of 21.4 mg (40%) assaying at 1.48 curies, 22.7 Ci/mmole, 69 mCi/mg. TLC: SiO_2-hexane 90, ethyl acetate 10, pyridine 0.1 elution showed the product (4a) at Rf of 0.60 at radiochemical purity of 91.3%. After adding 21 μL of pyridine, this material is stored in 100 mCi lots in 100 mL of toluene, under argon, in a low-temperature freezer.

Retinol-11,12-$^3H_2$(5). A solution of ca. 1.4 mg, 100 mCi, of 4a, prepared above, in 3.38 mL of toluene was added to a mixture of 6 mL of toluene, 1 mL of ethanol (super-dry), and 5 μL of 10N sodium hydroxide solution, all under argon. The resulting mixture was stirred at room temperature for 45 minutes then 3 μL of acetic acid was added and the mixture was concentrated to near dryness and chromatographed immediately over 14 mL of alumina (Woelm, neutral, activity grade III), packed in and eluted with hexane 80, ethyl acetate 20, pyridine 0.01. Fractions of 4 mL were taken and one drop of 100 ppm BHA/toluene was placed in each tube before collection. Fractions 13-17 were combined and concentrated to yield 58 mCi (58%) at specific activity of 22.7 Ci/mmole, 79 mCi/mg.[16] By tlc, SiO_2-hexane 70, ethyl acetate 30, pyridine 0.1, the product had Rf of 0.30 and radiochemical purity of 95%.

Retinoic-11,12-$^3H_2$ acid (6). An 18.6 mg (1.3 curie) portion of 4a stored in toluene solution was concentrated at 30°/10 mm and the residue dissolved in 4.6 mL of methanol (distilled from magnesium) and transferred, with two 0.5 mL rinses, to a stirred suspension of 35 mg of activated manganese dioxide and 93 mg of silver oxide (Ag_2O) in a mixture of 1.8 mL of water, 1.8 mL of methanol and 0.33 mL of 1N sodium hydroxide solution at 60°, under argon. The mixture was stirred under argon at 60° for 2 hr, then cooled, filtered, and the precipitate rinsed with three 1 mL portions of methanol all directly into a flask containing 11 mL of ice-cold water, 614 μL of 3M phosphoric acid and 5.6 mL of methylene chloride, with stirring, under argon. The phases were separated and the aqueous layer was extracted with three portions of methylene chloride, each of 5.6 mL. These were combined with the original organic layer and the total was washed with 5.6 mL of water containing 5 drops of brine, then dried over anhydrous magnesium sulfate,

filtered, and concentrated in vacuo to a residue of 19 mg which was taken up in 50 mL of toluene containing 10 μg of BHA and 10 μL of pyridine and stored overnight, under argon, at -15°. The solution was then concentrated to near dryness at 30°/10 mm and the residue chromatographed over 18 g of silica gel (E. Merck #9385) using cyclohexane 60, anhydrous ether 40, acetone 2, acetic acid 1 as the packing and eluting solvent. Fractions of 6 mL were collected into tubes containing 2 drops each of BHA solution (100 ppm/toluene). Fractions 6-8 were combined and concentrated in vacuo to a residue of 8.5 mg which was crystallized from 250 μL of toluene plus 1 mL of hexane. A first crop of 2.6 mg, 195 mCi (15%) at 22.7 Ci/mmole (75 mCi/mg) was obtained which by tlc, SiO_2-cyclohexane 60, anhydrous ether 40, acetone 2, acetic acid 1, had a single spot at Rf 0.55 corresponding exactly to an authentic sample of non-labeled all-trans retinoic acid. The radiochemical purity was 99.2%. The product was stored at 1 mCi/mL of toluene containing 40 μg of BHA and 4 μL of pyridine, under argon, at -60°.

Retinyl-11,12-$^3H_2$ propionate (4b). A 5 mg sample of 5 (24.5 Ci/mmole) was dissolved in 0.5 mL of toluene and treated with 0.2 mL of a toluene solution containing 18.8 μL of triethylamine and 17.7 μL of propionic anhydride. With magnetic stirring, under an atmosphere of nitrogen, the mixture was allowed to stand for 60 hours. The mixture was then cooled to 0°, treated with 0.5 mL of 1M sodium carbonate solution and stirred for 3 hr. The phases were separated and the organic layer was washed successively with 2 mL of water and of brine then dried over anhydrous magnesium sulfate, filtered and concentrated in vacuo to a residue which was chromatographed over 4.5 g of silica gel (E. Merck #9385) using hexane 90, anhydrous ether 10, and pyridine 0.01 as the packing and elution solvent. Fractions of 1 mL were taken and numbers 5-10 were combined and concentrated in vacuo to a residue of 0.96 mg having specific activity of 24.49 Ci/mmole and radiochemical purity (tlc) of 94.4%. The NMR spectrum of a non-labeled sample of retinyl propionate, prepared exactly as described above, was compatible and the molecular ion (m/e) was equal to 342.

Retinyl-11,12-$^3H_2$ myristate (4c). A 4.9 mg sample of 5 (16 Ci/mmole) was dissolved in 0.2 mL of heptane and treated with 5.4 mg of pyridine dissolved in 0.2 mL of heptane containing 8.4 mg of myristoyl chloride. The resulting mixture, under an argon atmosphere, was magnetically stirred at room temperature for 16 hr. To the mixture, 0.2 mL of 1N hydrochloric acid was added and the phases were separated. The organic layer was washed with 0.2 mL of water then 0.2 mL brine and then dried over anhydrous magnesium sulfate. The mixture was then filtered and the filtrate applied to 10 g of silica gel (E. Merck #9385) packed in and eluted with hexane 40, ethyl acetate 1 containing 0.5 mL each of pyridine and BHA (100 ppm/toluene) per liter. Fractions of 5 mL were collected and numbers 7 and 8 were combined and concentrated in vacuo to a residue of 4 mg (16 Ci/mmole) having radiochemical purity of 96.1% (tlc). The NMR spectrum of a non-labeled sample of retinyl myristate, prepared exactly as described above, was compatible.

Retinyl-11,12-$^{3}H_2$ palmitate (4d). A 4.9 mg sample of 5 (16 Ci/mmole) was dissolved in 0.2 mL of heptane and treated with a solution of pyridine in heptane (5.4 mg/0.1 mL) and a solution of palmitoyl chloride in heptane (9.35 mg/0.1 mL). Reaction and work-up were carried out exactly as described above for the preparation of 4c and yielded 3 mg of product (16 Ci/mmole) at 97.6% radiochemical purity. The material was stored under argon, at -60° in toluene (1 mCi/mL) containing 40 μg of BHA and 4 μL of pyridine. After 1 year, about 60% decomposition was noted, presumably due to radiolysis.

Methyl 2-bromoacetate-2-^{14}C (7). Bromoacetic-2-^{14}C acid was prepared by standard procedures[12] on a 2.88 mmole scale and was esterified, in ether solution, with diazomethane. Volatile material, including excess diazomethane, was removed at aspirator vacuum to yield the product, which was not further purified.

5-Hydroxy-4-methyl-2(5H)-furanone-3-^{14}C (8). Sufficient non-labeled methyl bromoacetate was added to that obtained above to provide a total of 2.5 mmoles. The material was dissolved in 10 mL of ether and 2.5 mmole (415.4 mg) of triethylphosphite was added. The mixture was stirred at 55° for 16 hours, cooled and the ether was removed by vacuum transfer. The ether solution that had transferred off was treated with a fresh portion of 1.25 mmole of triethylphosphite which was then stirred at 55° for 8 hr. Again, the transferred ether solution was treated in a similar manner with 0.63 mmole of fresh triethylphosphite. The residues from these 3 runs were combined and purified by molecular distillation to yield about 2.5 mmole of methyl diethylphosphonoacetate-2-^{14}C which was dissolved in 3.75 mL of dimethylformamide. The solution was treated with 3 mmole (0.365 mL) of pyruvic aldehyde dimethylacetal. Under a nitrogen atmosphere, the resulting mixture was warmed to 35° and treated with 2.75 mL of 1N sodium methoxide in methanol added dropwise over a 30 min period. The mixture was then stirred at room temperature for 4 hr, diluted with 25 mL of water and extracted with four 10 mL portions of ether. These were combined, washed with a few mL water, then brine, dried over anhydrous magnesium sulfate, filtered and concentrated in vacuo. The residue was treated with 5 mL of 6N hydrochloric acid then heated at reflux temperature for 4 hr. After cooling, the mixture was concentrated in vacuo, treated with water and again concentrated. This was repeated several more times, and finally, the residue was distilled to yield 285 mg (2.5 mmole, 36.8 mCi/mmole) of 8 with b.p. of 125°/110 μ. Lit[9], 90-92°/70 μ. The radiochemical yield from sodium acetate-2-^{14}C is about 75%.

13-cis-Retinoic-14-^{14}C acid (10). In a 10 mL round bottom 2-necked flask, were combined, under nitrogen, 276 mg (0.55 mmole) of the "C-15 Wittig" reagent 9 (as the phosphonium chloride), 56.7 mg (0.5 mmole, 18.4 mCi) of the butenolide 8 (obtained above) and 3 mL of 2-propanol. The stirred solution was chilled to -30° and treated with 0.3 mL of 1.75N potassium hydroxide in 2-propanol, dropwise, followed by a 0.3 mL portion added at once. The resulting mixture was allowed to warm to -5° over a 0.5 hr period and then stirred an additional 10 min at 0°. The mixture was poured into 50 mL of ice-water to which 10 drops of 1N sodium hydroxide solution had been added. Under

nitrogen, the mixture was extracted with 3 portions of ether (25 mL each) backwashing each with 5 mL of water then 1 mL of brine. The aqueous phases were combined and acidified to pH 4 with 0.3 mL of 10% sulfuric acid and then extracted, under nitrogen, with three 25 mL portions of ether. These were combined, washed with 5 mL of water then two 5 mL portions of brine then dried over anhydrous magnesium sulfate, filtered and concentrated in vacuo. The residue was redissolved in ether and chromatographed over 5 g of silica gel (E. Merck #7734) packed in ether. Elution with 1% acetic acid in ether yielded a 15 mL fraction containing mixed isomers of retinoic acid. This was concentrated in vacuo to a 90 mg residue (5.6 mCi) which was dissolved in 3 mL of ether and treated with 0.5 mL of 1.0 mg/mL of iodine in ether for 1.5 min at room temperature in the light. A 0.5 mL portion of 5% sodium thiosulfate solution was added and the mixture was then washed successively with 0.5 mL water, 0.5 mL brine, dried over anhydrous magnesium sulfate, filtered and concentrated in vacuo to a yellow solid residue of 88 mg. This was crystallized by the addition of 0.3 mL of methanol, with warming, followed by refrigeration. The supernatant was removed and the crystals were washed with 0.15 mL of methanol and then twice with 0.15 mL each of pentane and then dried to a constant weight of 42 mg (3.15 mCi). Radiochemical purity was found to be greater than 99% (tlc on silica gel; cyclohexane 60, ether 40, acetone 2, acetic acid 1 elution, Rf=0.65).

[3-Methyl-5-(2,6,6-trimethylcyclohex-1-enyl)-2,4-pentadienyl]-1,2-$^{14}C_2$ triphenylphosphonium bromide (15c). Acetylene-1,2-$^{14}C_2$ was prepared from 2.15 mmole (118 mCi) of barium carbonate-^{14}C via barium carbide-^{14}C as described in the literature.[13] By vacuum transfer, the acetylene was added to a mixture of n-butyl lithium, 1 mmole (1.44M/hexane), in 5 mL of DMF to which had been added 2.0 mmole of diethylamine. The resulting mixture was stirred under vacuum at -78° for 45 min then, under an atmosphere of argon, 1 mmole (192.3 mg) of beta ionone (12) in 1 mL of tetrahydrofuran was added. The resulting mixture was stirred under argon for 1 hr as the bath temperature was permitted to rise from -78° to -55° and for an additional 45 min as the bath temperature rose to -10°. About 20-30 mg of solid ammonium chloride was then added and after 10 min, 5 mL of ammonium chloride saturated water was added. The resulting mixture was extracted with 2 fifteen mL portions of ether which were combined and dried over anhydrous magnesium sulfate. After filtration, the solution was concentrated in vacuo to a residue of 218 mg (99% as ethynyl beta ionol) having 89% radiochemical purity (tlc, silica gel, hexane 4, ether 1 elution. Rf 0.33). This material was chromatographed over 50 g of alumina (Woelm, activity III) packed in hexane and eluted gradiently with hexane to hexane 4, ether 1. Fractions of 20 mL were taken and combining 28-37 afforded 119 mg (0.54 mmole, 60 mCi) after evaporation.

The chromatographed product was dissolved in 5 mL of hexane and treated with 20 mg of freshly prepared Lindlar vitamin A catalyst and 10 μL of quinoline. An atmosphere of hydrogen was introduced and the resulting mixture was magnetically stirred at 0° for 35 min (hydrogen uptake of 12.5 cc was noted at 28 min). The mixture was then filtered through Celite, the precipitate was washed with six 1 mL portions of hexane which were

combined with the original filtrate, dried over anhydrous magnesium sulfate, filtered and concentrated in vacuo to a residue of 104 mg (0.465 mmole) of vinyl beta ionol. Without further purification, this material was dissolved in 1 mL of methanol and 183 mg (0.535 mmole) of triphenylphosphine hydrobromide was added. Under nitrogen, the resulting mixture was stirred at room temperature for 25 hr. A 5 mL portion of 2-propanol was added and the mixture was concentrated in vacuo. A 10 mL portion of 2-propanol was added and again the mixture was concentrated. The residue was slurried with three 5 mL portions of ether which were removed by decantation and then dried in vacuo to a residue of 286 mg (0.53 mmole, 60 mCi) which was diluted with non-labeled 15 to specific activity of 30 mCi/mmole.

13-cis-Retinoic-10,11-$^{14}C_2$ acid (10c). An 0.83 mmole portion of phosphonium salt (15c), obtained above, was treated with an 0.87 mmole portion of non-labeled butenolide (8) as described above for the preparation of 10. After chromatography of the retinoic acid isomers, isomerization[14] was effected by dissolving the isomeric mixture in 0.4 mL THF and adding 1.2 mL of acetonitrile. A 0.2 mL portion of catalyst solution (prepared by adding 11 mg palladium nitrate, 51 mg triphenylphosphine, and 13.5 μL of triethylamine to 5 mL of acetonitrile) was added and the resulting mixture was rapidly heated to 50° where stirring was maintained for 1 hr. Then, 1.2 mL of water was added, the mixture was cooled to 0° and stirring was continued for an additional 2 hr. The mixture was filtered and the crystals were washed with ice-water (three 0.5 mL portions) then 3 portions, each of 1 mL, of cold acetonitrile and then dissolved in methylene chloride. This solution was dried over anhydrous magnesium sulfate, filtered and concentrated in vacuo to a residue of the desired product, 10c, 154 mg (0.51 mmole) with radiochemical purity of 95% (tlc). The product may be further purified by crystallization from ethyl acetate-hexane to give product with 97.4% radiochemical purity and specific activity of about 100 μCi/mg.

[3-Methyl-5-(2,6,6-trimethylcyclohex-1-enyl)-2,4-pentadienyl]-1,2-$^2H_2$-triphenylphosphonium bromide (15a). This was prepared from ethynyl beta ionol (13) by partial reduction under an atmosphere of deuterium as described above for the preparation of the carbon-14 labeled analog. Conversion to the salt (15a) was carried out as described above for the preparation of the carbon-14 labeled analog (15c).

13-cis Retinoic-10-^2H acid (10a). This was prepared from the dideutero Wittig salt (15a) on a 0.27 mmole scale as described for the preparation of 10c. The product had d_1 content=100% (mass spectrum).

13-cis-Retinoic-10-^3H acid (10b). On a scale of 0.37 mmole (81 mg) of ethynyl beta ionol (13), selective reduction was effected with 20 Ci (0.33 mmole) of carrier free tritium gas as follows. The alcohol (13) was dissolved in 3 mL of hexane and then treated with 5.7 μL of quinoline then with 11.5 mg of Lindlar vitamin A catalyst. The system was evacuated and degassed, then the tritium gas was admitted by means of a Toeppler pump. At 0°C, the mixture was stirred for 85 min, then any unreacted tritium was removed and an atmosphere

of hydrogen was introduced and again the mixture was stirred at 0° for 30 min. After this time, the mixture was filtered through Celite and the Celite was thoroughly washed with hexane. The combined hexane solutions were concentrated in vacuo and the residue was treated with 3-4 mL of methanol which was then removed by vacuum transfer. This process was repeated to insure complete removal of labile tritium and yielded a residue of product, 12.12 Ci, which by tlc on silica gel, ethyl acetate 1, hexane 4 elution showed a single spot at Rf 0.58 and had radiochemical purity of 99%. After storage overnight in toluene solution, under nitrogen, at -60°, the material was converted to the tritium labeled triphenylphosphonium salt (15b) as described above for the preparation of the carbon-14 analog 15c, to provide 7.95 Ci of product with specific activity of about 21.5 Ci/mmole. This was diluted with an approximately equal amount of unlabeled 15 to afford a total of 0.76 mmole which was converted to the desired product as described above for the preparation of 10c. Dilution with non-labeled product was effected to improve the overall yield which was 32.7 mg (0.109 mmole) at 16.2 mCi/mg for a total of 531 mCi of crystalline 10b having radiochemical purity of 95.5%. It is again noted that one tritium atom of the Wittig salt (15b) is lost during the conversion to the ylid which is carried out in a protic solvent (2-propanol) and effectively halves the specific tritium activity of the product.

Approximately 500 mCi of 10b was stored at -60° in an evacuated flask where it underwent considerable radiolysis after 3-4 months. After 9 months of storage, the material was repurified by recrystallization from ethyl acetate and hexane followed by flash chromatography over fine silica gel using cyclohexane 60, ether 40, acetone 2, pyridine 0.1, acetic acid 1 and antioxidant solution 0.1 as eluting solvent. About 50 mCi of pure product was obtained in this manner.

13-cis-Retinoic 8, (9,9,9-methyl) d_4 acid (10d). Beta ionone-d_4 was prepared from citral and acetone-d_6 as previously[7] described with the exception of using deuterated reagents and solvents in this instance. The preparation was carried out on a scale of 10 mmole of citral and excess acetone-d_6 and yielded 70% of the desired product (12a). Conversion to ethynyl beta ionol and vinyl beta ionol were carried out as described above for deuterium or carbon-14 labeled analogs. Subsequent reactions to the desired product were also carried out as described above. The final condensation was carried out on one mmole of the tetradeuterated Wittig salt and yielded 44.8 mg of crystalline product (10d) which was d_4=94% (mass spectrum).

4-Oxo-13-cis-retinoic-8,9,19-$^{13}C_3$ acid (20). Beta ionone-$^{13}C_3$ was prepared from citral and acetone-1,2,3-$^{13}C_3$, in the usual manner, on a 1 mmole scale. The product was dissolved in 0.2 mL of DMSO and placed in a 0.5 mL microsyringe. A 474 mg (2.2 mmole) portion of pyridinium chlorochromate was dissolved in 0.8 mL DMSO and the solution was placed in a 2 mL pipette. The contents of both the pipette and the microsyringe were simultaneously added over 1 hr to 0.2 mL DMSO which was magnetically stirred, under nitrogen, at 100°. The mixture was then stirred at 100° for an

additional 4 hr then cooled and extracted with 6 two mL portions of ether. The combined ether extracts were washed successively with 5 mL each of water, 0.1N hydrochloric acid, water and brine. The combined DMSO-aqueous phase was diluted with 10 mL of 0.1N hydrochloric acid and extracted with 10 mL of ether. This ether phase was treated exactly as the first one then the two ether phases were combined, dried over anhydrous magnesium sulfate, filtered and concentrated under reduced pressure to a residue of 145 mg. The residue was chromatographed over 40 g of silica gel 60 with ether 4, hexane 6 as the eluting solvent. From this chromatography, 31.6 mg of unreacted beta ionone-$^{13}C_3$ was isolated along with 77 mg of 4-oxo-beta ionone-$^{13}C_3$. The beta ionone-$^{13}C_3$ was resubjected to the oxidation procedure and yielded an additional 17.7 mg of 4-oxo-beta ionone-$^{13}C_3$ for a total of 94.7 mg (0.45 mmole, 45%) of product (19).

Reaction of 19 with acetylene was carried out under the same conditions described above for other labeled forms of ethynyl beta ionol. The addition of acetylene took place exclusively at the acyclic ketone and afforded 120.8 mg of product on work-up. Partial reduction to 4-oxo-vinyl beta ionol proceeded as described above and provided 73 mg of product after chromatographic work-up. Conversion to the Wittig salt and subsequent reaction with butenolide were carried out as described in the literature.[15] After palladium nitrate isomerization, chromatography, and crystallization, 26.4 mg of the desired product (20) was obtained. The NMR spectrum was compatible.

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