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SYNTHESIS OF 10,11,14,15-$^{13}C_4$- AND 14,15-$^{13}C_2$-RETINYL ACETATE

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SUMMARY

The synthesis of 10,11,14,15-$^{13}C_4$- and 14,15-$^{13}C_2$-retinyl acetate is described. The procedure uses a modified Wittig-Horner synthesis. By using the ^{13}C isotope, sensitive gas chromatography-combustion-isotope ratio mass spectrometry can be used for analysis in application to human isotope dilution studies.

Keywords: ^{13}C-labelled vitamin A analogs, ^{13}C-retinyl acetate, 10,11,14,15-$^{13}C_4$-retinyl acetate, 14,15-$^{13}C_2$-retinyl acetate, retinoid synthesis, gas chromatography-combustion-isotope ratio mass spectrometry

INTRODUCTION

The synthesis of deuterated forms of vitamin A has previously been described (1). Tetra-deuterated retinyl acetate has been applied to human studies for use in isotope dilution assays to determine total body reserves of vitamin A (2,3,4). In this assay conventional GCMS is used and therefore relatively large doses of the deuterated compound are administered to humans (35-140 μmole). The retinol also needs to be derivatized before analysis. Gas chromatography-combustion-isotope ratio mass spectrometry (GCCIRMS) has been applied in studies using uniformly labelled ^{13}C-β-carotene (5,6). GCCIRMS offers more sensitivity than conventional GCMS. This prompted us to synthesize ^{13}C labelled retinyl acetate for use with GCCIRMS in isotope dilution assays to assess vitamin A status. Retinoids and

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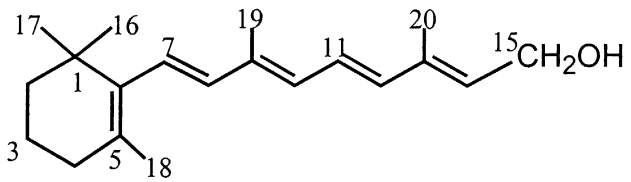
carotenoids are primarily synthesized by modification to the Wittig reaction (1, 7-9). Scheme 1 outlines the numbering system used for retinol.

Scheme 1



Numbering system for retinol

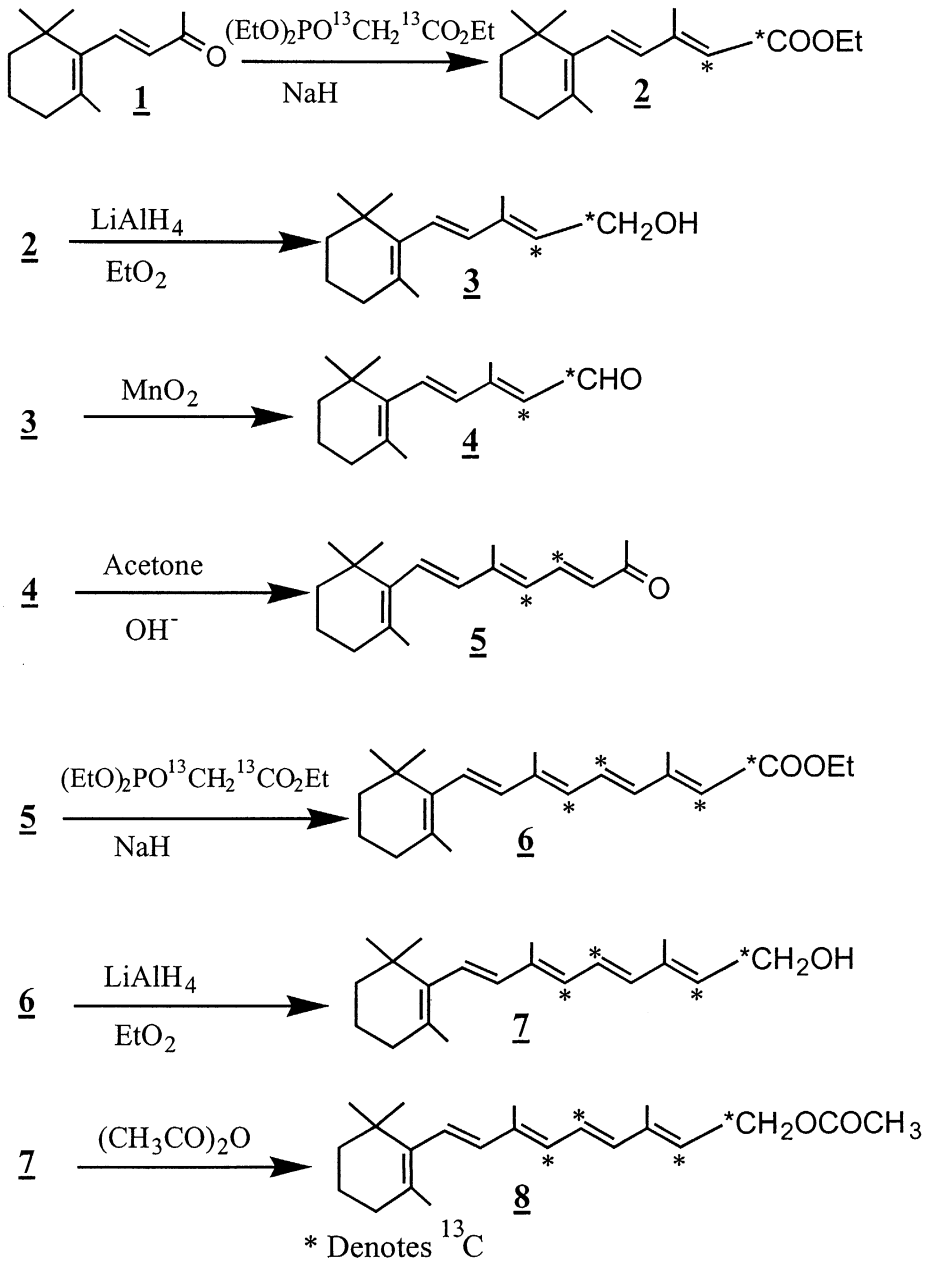
After determining the natural abundance of ^{13}C in retinol at baseline, the $^{13}C_4$-retinyl acetate was used in a controlled rat experiment (10). Because more than adequate sensitivity was achieved using physiological doses of $^{13}C_4$-retinol, we have also synthesized $^{13}C_2$-retinyl acetate for future studies. The synthetic advantages of using 2 instead of 4-^{13}C include improved yields and substantial cost saving.

RESULTS AND DISCUSSION

The synthesis of 10,11,14,15-$^{13}C_4$-retinyl acetate (Scheme 2) is accomplished by using modified Wittig-Horner reactions. In this regard, the carbanion of commercially available $^{13}C_2$-triethylphosphonoacetate (Aldrich) is added to β-ionone (Aldrich) {1} to make ethyl-β-ionylidene acetate {2}. 2 is reduced to β-ionylidene ethanol {3} and oxidized to β-ionylidene acetaldehyde {4}. Under basic conditions, acetone is added to 4 to yield C-18 tetraene ketone {5}. The carbanion of $^{13}C_2$-triethylphosphonoacetate is added to 5 to give 10,11,14,15-$^{13}C_4$-retinoic acid ethyl ester {6}. 6 is reduced to 10,11,14,15-$^{13}C_4$-retinol {7} and acetylated to 10,11,14,15-$^{13}C_4$-retinyl acetate {8}. In the synthesis of 14,15-$^{13}C_2$-retinyl acetate, unlabelled triethylphosphonoacetate is used in the first 2-carbon addition to 1.

The yields at each step are good. The biggest challenge in the preparation of the retinyl acetate is separation of *cis/trans* isomers. Silica gel worked well for the separation of *cis/trans* isomers of C-18 tetraene ketone, giving >90% all-*trans* after purification. The purification of the C-18 tetraene ketone was essential to success of the overall synthesis. For separation of *cis/trans* isomers of retinyl acetate, deactivated neutral alumina columns were useful; however, a large amount of

Scheme 2

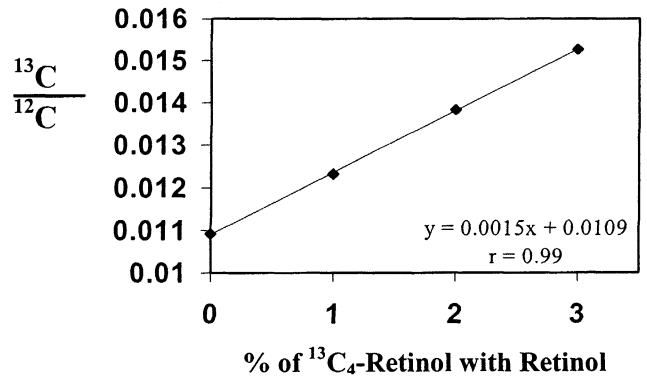


cis/trans isomers coeluted when quantities >1 g were purified on relatively large columns (400 g). From an overall yield of 4.5 g crude *cis/trans* retinyl acetate, 0.5 g was obtained after purification of >96% all-*trans* retinyl acetate, suitable for use in animal and human studies.

The synthesis of 14,15-$^{13}C_2$-retinyl acetate has advantages over the synthesis of 10,11,14,15-$^{13}C_4$-retinyl acetate: cost and yield. Although the basic chemical steps are the same between the $^{13}C_4$ and $^{13}C_2$ compounds, excess intermediates are made in the $^{13}C_2$ synthesis to optimize the yield of labelled product.

When solutions of 0-3% of $^{13}C_4$-retinol in unlabelled retinol were directly injected into the GCCIRMS the relationship was indeed linear (Figure 1, r = 0.99). This level of enrichment is what would be expected in individuals who are dosed with physiological amounts of 10,11,14,15-$^{13}C_4$-retinyl acetate and 14,15-$^{13}C_2$-retinyl acetate.

Figure 1



EXPERIMENTAL

All laboratory procedures were carried out under gold fluorescent lighting to limit photo degradation and isomerization. Intermediates were purified on 4% water deactivated silica gel. Final products were purified on 8% water deactivated neutral alumina (Aldrich). Reactions were monitored by using thin-layer chromatography on Machery-Nagel silica gel plates (Bodman) with 80:20 hexanes:ethyl acetate as eluant and UV illumination (254 and 365 nm). After reaction completion, the TLC plates were exposed to iodine gas to visualize products and impurities. Dilute mixtures of 0, 1, 2 and 3% $^{13}C_4$-retinol in unlabelled retinol were analyzed with a 5890A Hewlett-Packard GC (Wilmington, DE) fitted with a Fisons VG Isotech Isochrom GC-combustion interface to the Fisons/VG Isotech Optima isotope ratio MS (Manchester, UK). The isotope ratio of each sample was determined by comparison with a working standard of CO_2 gas.

Synthesis of 10,11,14,15-$^{13}C_4$-retinyl acetate

Sodium hydride (NaH, 355 mg, 0.015 mole) was covered with 20 mL of ether. $^{13}C_2$-triethylphosphonoacetate (2.96 g, 0.013 mole) in 10 mL ether was added dropwise and stirred for 2 h. H_2 was released and the initial suspension became clear and amber. β-ionone (2.3 g, 0.012 mole) {1} in 10 mL of ether was added and reacted overnight. The next morning 1g of $^{13}C_2$-triethylphosphonoacetate was reacted with 135 mg of NaH and added to the reaction. On day 2, the reaction was quenched with water and the ethyl-β-ionylidene acetate {2} was extracted into hexane and washed with water. The organic layer was dried ($MgSO_4$), filtered and concentrated with a rotary evaporator to yield 3.2 g 2 (>99% yield).

Lithium aluminum hydride ($LiAlH_4$, 700 mg, 0.019 mole) was stirred in anhydrous diethyl ether on a dry-ice acetone bath. 2 (3.2 g, 0.012 mole) was dissolved in 50 mL of ether and added dropwise to the $LiAlH_4$. The reaction was stirred for 1 h and warmed to room temperature. The reduction was monitored by TLC until complete. Ice chips were added to deactivate excess $LiAlH_4$. β-ionylidene ethanol {3} was extracted with diethyl ether and washed with water. 1 N H_2SO_4 was used to dissolve the aluminum hydroxide precipitate. The organic layer was dried ($MgSO_4$), filtered and concentrated to yield 3.0 g (0.013 mole) 3 (>99% yield).

3 (3.0 g, 0.013 mole) was dissolved in 100 mL hexane and oxidized with manganese oxide (MnO_2, BDH) overnight. Four aliquots of 5 g each of MnO_2 were added during the next 24 h. After oxidation to β-ionylidene acetaldehyde {4} was deemed complete by TLC, MnO_2 was removed and washed with dichloromethane. The filtrate was rotary-evaporated to yield 2.7 g of 4 (0.012 mole, 92% yield).

4 (2.7 g, 0.012 mole) was dissolved in 75 mL of acetone, treated with 5 mL of 1 N NaOH, and stirred for 6 h. C-18 tetraene ketone {5} was extracted with hexane and washed with water. The resultant solution was dried and rotary evaporated to yield 3 g of crude *cis-trans* 5 (0.011 mole, 90% yield). Because the *trans* isomer is most desirable for human studies, the mixture was purified on 4% water deactivated silica gel (300 g) using a slow gradient of 0-10% ethyl acetate in hexane. After purification, 1.2 g of 5 was obtained (90% all-*trans*).

NaH (225 mg, 0.0095 mole) was stirred with 20 mL of ether. $^{13}C_2$-triethylphosphonoacetate (1.95 g, 0.0086 mole) in 10 mL ether was added dropwise and stirred for 2 h. 5 (1.2 g, 0.0044 mole) in 10 mL ether was added and reacted overnight. The reaction was quenched with water and the 10,11,14,15-$^{13}C_4$-retinoic acid ethyl ester {6} was extracted with hexane and washed with water. The organic layer was dried and concentrated to a crude yield of 1.6 g 6. 6 was purified on 8% water deactivated neutral alumina (300 g) with a slow 0 to 3% diethyl ether in hexane gradient with a yield of 0.8 g *cis/trans* isomers.

10,11,14,15-$^{13}C_4$-Retinol {7} was prepared by adding 0.8 g 6 to 125 mg of $LiAlH_4$ stirring in ether on a dry ice-acetone bath. After 1 h, the reaction mixture was warmed to room temperature. If the reaction was incomplete, the solution was cooled and more $LiAlH_4$ added. After completion as judged by TLC, the reaction was quenched by adding ice chips to deactivate remaining $LiAlH_4$. 7 was extracted with ether, washed with water, dried ($MgSO_4$) and evaporated to yield 0.8 g wet weight. 7 was cooled in an ice bath and dissolved in triethylamine:acetic anhydride (3 mL:1 mL). After 2 h, 10,11,14,15-$^{13}C_4$-retinyl acetate {8} was extracted with hexane, washed with water, dried ($MgSO_4$) and evaporated. The crude mixture was purified on 8% water deactivated neutral alumina (250 g). After purification, 150 mg of 10,11,14,15-$^{13}C_4$-retinyl acetate was obtained: 70 mg of 75:25 *cis:trans* and 80 mg of >93% *trans* as judged by reversed phase HPLC. The MW of 332 was confirmed by conventional GCMS with on-column injection and EI detection.

Synthesis of 14,15-$^{13}C_2$-retinyl acetate

NaH (2 g, 0.08 mole) was covered with 50 mL of ether. Unlabelled triethylphosphonoacetate (18 g, 0.08 mole) in 50 mL ether was added dropwise and stirred for 2 h. H_2 was released and the initial suspension became clear and amber. β-ionone (10.3 g, 0.057 mole) in 20 mL of ether was added and reacted overnight. On day 2, the reaction was quenched with water and the ethyl-β-ionylidene acetate (2) was extracted into hexane and washed with water. The organic layer was dried ($MgSO_4$), filtered and concentrated to yield 14.6 g 2 (99% yield). TLC detected only minor impurities.

2 (14.6 g, 0.057 mole) was reduced with 2.3 g (0.06 mole) $LiAlH_4$ in ether over a dry-ice acetone bath yielding 11.6 g of β-ionylidene ethanol {3} (93%). 3 (11.6 g,

0.053 mole) was oxidized with MnO_2 (75 g over two nights) to give 10.2 g (0.048 mole) of β-ionylidene acetaldehyde {4} (90% yield). 4 was treated with acetone under basic conditions to make C-18 tetraene ketone {5}. 5 was carefully purified on 4% water deactivated silica gel and 11 g was recovered (>90% all-*trans*). 5 (11 g) was reacted with the carbanion of $^{13}C_2$-triethylphosphonoacetate (11 g) to give 13 g of crude 14,15-$^{13}C_2$-retinoic acid ethyl ester {6}. 6 was purified on 8% water deactivated neutral alumina to give 5 g of *cis/trans* isomers. 6 was subsequently reduced to 14,15-$^{13}C_2$-retinol {7} and acetylated to 8 by dissolving in triethylamine:acetic anhydride (40 mL:4 mL). The reaction was kept at room temperature for 2 h and then refrigerated overnight. After extraction into hexane, the overall crude yield was 4.5 g. Purification on 8% water deactivated neutral alumina gave 0.5 g of >96% all-*trans* 14,15-$^{13}C_2$-retinyl acetate. The final product was characterized using TLC (one spot detected), scanning UV-VIS spectroscopy and microbore reversed-phase HPLC (3.5-μm, C_{18} Waters Symmetry 7.5-cm column) with photo-diode array detection scanning from 250 to 400 nm.

CONCLUSION

The synthesis of 10,11,14,15-$^{13}C_4$-retinyl acetate and 14,15-$^{13}C_2$-retinyl acetate was accomplished using a 7-step procedure beginning with commercially available β-ionone. Labelling was performed by using a modified Wittig-Horner procedure with commercially available $^{13}C_2$-triethylphosphonoacetate. The compounds are to be used in an isotope dilution assay to determine total body reserves of vitamin A. The method utilizes GCCIRMS for analysis of retinol purified from serum of individuals who have been dosed with physiological levels of the labelled compounds.

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