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Research Article

Synthesis of tracer labelled [11,12-^{3}H]-β-carotene

Bhabesh C. Goswami[†] and Arun B. Barua*
Department of Biochemistry, Biophysics & Molecular Biology, Iowa State University, Ames, IA 50011, USA

Summary

The synthesis of tracer labelled [11,12-^{3}H]-β-carotene is described. The procedure uses Wittig condensation of tracer labelled ^{3}H-retinal (retinal spiked with [11,12-^{3}H]-retinal) with retinyl triphenylphosphonium bromide. The preparation of tracer labelled[^{3}H]-β-carotene is suitable for studies involving bioavailability and bioconversion of β-carotene to vitamin A.

Key Words: tritium; [11,12-^{3}H]-β-carotene; retinol; retinal; vitamin A

Introduction

Vitamin A-deficiency is a public health problem in many regions of the world. Preformed vitamin A can be obtained from animal sources. Vitamin A can also be biosynthesized in the body from dietary provitamin A carotenoids, β-carotene in particular. However, for reasons still unknown, bioavailability of carotenoids is not as efficient as was thought previously. Since it is impossible to make animals and humans completely free from vitamin A, it is difficult to quantify any

[†]Present address: Department of Chemistry, Guahati University, Guwahati 781014, India.
*Correspondence to: A. B. Barua, Department of Biochemistry, Biophysics & Molecular Biology, 3256 Molecular Biology Building, Iowa State University, Ames, IA 50011, USA. E-mail: abarua@iastate.edu

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newly absorbed β-carotene and newly formed vitamin A after ingestion of a given amount of β-carotene. Accurate information about the bioavailablity of β-carotene and newly formed vitamin A should be possible by use of a label in the β-carotene molecule.

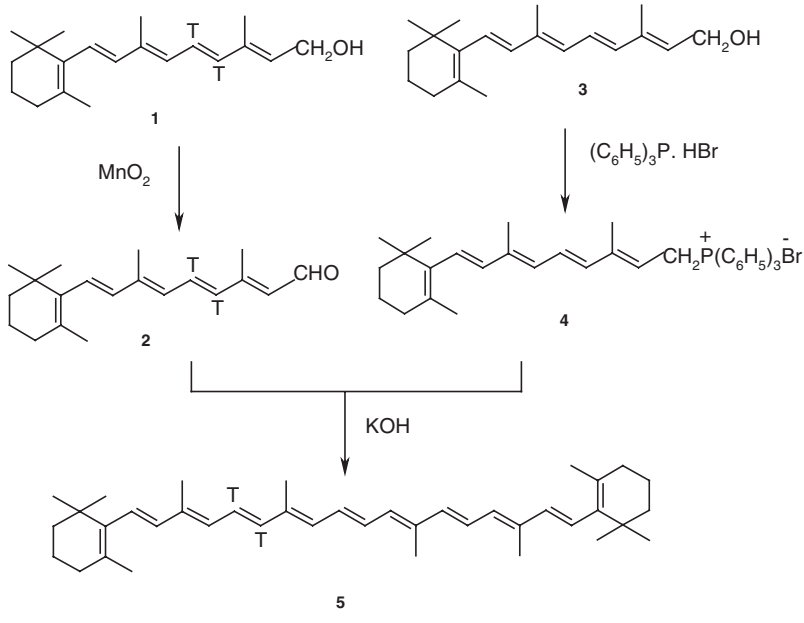
Carotenoids labelled with a radioisotope like ^{3}H or ^{14}C have been prepared from a labelled precursor by biosynthesis in various organisms and plants.[1] Starting from [2-^{14}C]-bromoacetic acid, synthesis of [6,6′-^{14}C]-β-carotene was described[2] in low yield. Synthesis of [15,15′-^{14}C]-β-carotene[3] was accomplished by use of radioactive acetylene according to the scheme $C_{19} + C_2 + C_{19} = C_{40}$. Radiolabeled retinoids or carotenoids have been prepared by reduction of carbonyl groups with NaB^3H_4/LiB^3H_4 or by partial hydrogenation of triple bonds to double bonds with $^3H_2$.[4–8] The synthesis of carotenoids specifically labelled with isotopic carbon and tritium has been reviewed.[9] The synthesis of ^{14}C-β-carotene[10] and all-*trans*-[10-^{3}H]-8′-apo-β-carotenoic acid[11] have been reported.

The $C_{20} + C_{20} = C_{40}$ scheme for the preparation of β-carotene has been utilized for preparation of β-carotene by way of the Wittig reaction, the Horner reaction, the Grignard reaction and dimerisation.[12] The synthesis of deuterated β–carotene has previously been reported from this laboratory[13] starting from tetradeuterated-β-ionylidene ethanol using the $C_{15} + C_{10} + C_{15} = C_{40}$ scheme. [11,12-^{3}H]-Retinol **(1)** is commercially available and can be easily oxidized to [11,12-^{3}H]-retinal **(2)**. Therefore, we considered the Wittig reaction suitable for the condensation of [11,12-^{3}H]-retinal with the triphenylphosphonium bromide salt of retinol to give [11,12-^{3}H]-β-carotene **(5)**. In this paper, we describe the chemical synthesis of tracer labelled [11,12-^{3}H]-β-carotene. We have used this preparation successfully in studying the bioavailability of β-carotene and its conversion to vitamin A in rats.[14]

Results and discussion

Following a modified Wittig reaction, we have synthesized tracer labelled [^{3}H]-β-carotene according to Scheme 1. The positions of ^{3}H incorporation are shown with the letter T.

Retinoids are unstable towards light, heat and air. To reduce losses of the tiny amount of the starting material, [11,12-^{3}H]-retinol, and for ease of handling, [11,12-^{3}H]-retinol was diluted with unlabelled retinol to give tracer labelled [11,12-^{3}H]-retinol. The first step involved the



Scheme 1. Synthesis of β-carotene enriched with [11, 12-^{3}H]-β-carotene

conversion of tracer labelled ^{3}H-retinol **(1)** to tracer labelled ^{3}H-retinal **(2)** (yield $\sim$77%) by MnO_2 oxidation. The second step was the preparation of the Wittig salt of retinol **(4)** by the equimolar addition of triphenylphosphine hydrobromide to a stirred solution of unlabelled retinol **(3)** in dry methanol (yield $\sim$86%). The third and final step was the Wittig reaction of the tracer labelled ^{3}H-retinal in dry ethanol with excess of Wittig salt of retinol in the presence of KOH to form tracer labelled ^{3}H-β-carotene **(5)**. Isolation and purification by TLC resulted in a pure preparation of tracer labelled [11,12-^{3}H]-β-carotene (specific activity 3.8×10^8 Bq/mmol, 10.3 mCi/mmol).

Experimental

All work with retinoids and carotenoids was performed in laboratories with yellow fluorescent lights (F40 Gold). The reactions were performed under argon. [11,12-^{3}H]-Retinol (250 μCi, 9.25×10^6 Bq) was supplied under argon in 125 μl EtOH containing 1 mg/ml D-α-tocopherol by New England Nuclear, Boston, MA. The specific activity of [11,12-^{3}H]-retinol was 49.7 Ci/mmol (1.84×10^{12} Bq/mmol). Crystalline synthetic all-*trans*-β-carotene and retinol were purchased from Sigma (St Louis,

MO). Anhydrous Et_2O, hexane, Ac_2O, KOH, EtOH, HPLC solvents (MeOH and CH_2Cl_2), and scintillation fluid (Scintiverse BD) were supplied by Fisher Scientific Co. (Fair Lawn, NJ). MnO_2 (precipitated) was obtained from British Drug Houses, Poole, England. Triphenylphosphine hydrobromide and dry MeOH were purchased from Aldrich (Milwaukee, WI). TLC was carried out on Polygram silica-gel G plates (4 cm × 8 cm, 20 cm × 20 cm, Macherey-Nagel, Duren, Germany) supplied by Brinkmann Instruments, Westbury, NY, according to the already described procedure[15] using a solvent mixture of hexane/acetone (9:1,v/v). Extremely dry MeOH and EtOH were obtained by refluxing commercial MeOH and absolute EtOH with Mg-turnings and I_2 followed by fractional distillation into a container having Type 4A molecular sieve.[16] The UV-Visible spectra were recorded on a Shimadzu (model UV 2101 PC) scanning spectrophotometer. The mass spectrum was recorded on a Finnigan TSQ700 triple quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) equipped with a Finnigan ESI interface. Radioactivity was counted in a Packard Scintillation counter (Model 1600 TR) for 2 min after vortexing 10–50 µl of the solution with 20 ml of Scintiverse BD in a 20 ml scintillation vial. The efficiency of tritium counts was $\geqslant 60\%$. The concentrations of retinol, retinal and β-carotene were determined from E (1%, 1 cm) of 1810, 1690 and 2592 (hexane), respectively.[17,18] Reversed phase high performance liquid chromatography (HPLC)[15,18] was performed on a Rainin C_{18} Microsorb-MV 3 µm (3.6 × 100 mm) column by using a linear gradient of MeOH/water (7:3,v/v) containing 10 mM ammonium acetate to MeOH/CH_2Cl_2 (4:1,v/v) at a flow rate of 0.8 ml/min. Identification of peaks was confirmed by examination of UV-Visible spectrum. The HPLC was equipped with a Waters (Milford, MA) PDA detector (model 996) and Millenium software and the separation was monitored at 445 nm.

*Oxidation of tracer labelled [11,12-^{3}H]-retinol to tracer labelled [11,12-^{3}H]-retinal (**2**)*

The solution of [11,12-^{3}H]-retinol **(1)** (250 µCi; ~1.4 µg, ~5 nmol based on specific activity) in ethanol was evaporated to dryness in a 10 ml glass tube under a stream of argon. Unlabelled retinol (3 mg, 0.01 mmol) was added to [11,12-^{3}H]-retinol. The residue of tracer labelled [11,12-^{3}H]-retinol was dissolved in hexane (5 ml). The solution was stirred with MnO_2 (16 mg) for 8 h under argon. The oxidation was monitored by TLC. When it was found that retinol (λ_{max} 325 nm) was completely

converted to retinal (λ_{max} 368 nm), stirring was stopped. The solution was centrifuged and the hexane solution was removed by means of a pipette. The residual pellet was broken, and extracted with hexane until the extracts showed no radioactivity. The pooled hexane extract was evaporated to dryness in vacuo. The oxidized product was purified by TLC on silica gel with 10% acetone in hexane to give tracer labelled [11,12-^{3}H]-retinal (2.3 mg, 77%).

*Preparation of retinyl triphenylphosphonium bromide (**4**)*

The procedure described by Surmatis[19] was used with some modifications for the preparation of the bromide salt and Wittig reaction. To a solution of retinol **(3)** (50 mg, 0.175 mmol) in dry MeOH (1 ml), triphenylphosphine hydrobromide (69 mg, 0.2 mmol) in MeOH was added dropwise with stirring. The mixture was stirred at room temperature under argon. After 1 h, the solvent was evaporated in a rotary evaporator (at about 38°C) and the light orange residue was washed with 5 ml portions of hexane 5–6 times to remove side products that showed λ_{max} 370, 350, 330 nm. TLC of the product on silica gel G plates with 10% acetone in hexane showed only one compound that did not move at all from the origin on the TLC plate. The TLC purified sample of retinyl triphenylphosphonium bromide (86.4 mg, 86%) showed λ_{max} at 343 in methanol.

*Wittig reaction: synthesis of tracer labelled [11,12-^{3}H]-β-carotene (**5**)*

To a stirred solution of 10 mg of retinyl triphenylphosphonium bromide in 2 ml of dry ethanol at −70°C, tracer labelled [11,12-^{3}H]-retinal (2.3 mg, 0.008 mmol) in 2 ml of dry ethanol was added dropwise. KOH (0.05 g) dissolved in dry ethanol (500 µl) was added slowly to the mixture, and the solution was stirred at −70°C for 15 min and then at room temperature for 2 h. An aliquot was tested by recording the visible spectrum and also by TLC on silica gel G. When no more carotene formation was noted after 2 h, water (1 ml) was added to the reaction mixture and the product was extracted with 3 ml portions of diethyl ether. The organic phase was removed under a flow of argon. The residue was dissolved in a few drops of CH_2Cl_2 and subjected to TLC. Tracer labelled ^{3}H-β-carotene separated very well as a dark orange band. The band was removed by scraping with a spatula, and the carotenoid was extracted with diethyl ether. The solvent from the

extract was evaporated using a flow of argon and the residue was dissolved in hexane. The amount of tracer labelled ^{3}H-β-carotene was estimated spectrophotometrically (2.0 mg, 67% yield with respect to 3 mg of retinol). The specific activity of the tracer labelled ^{3}H-β-carotene was determined to be 3.8×10^{8} Bq/mmol (10.3 mCi/mmol). HPLC of an aliquot showed that no *cis*-isomer of β-carotene was present.

HPLC of tracer labelled [11,12-^{3}H]-β-carotene

The purity of the TLC purified sample was ascertained by injecting standard β-carotene spiked with tracer labelled ^{3}H-β-carotene onto a C_{18} reversed phase column. It was found that the mixture coeluted as a single peak. As the compound prepared did not have any *cis*-peak in its UV visible spectrum and the retention time was the same as that of all-*trans*-β-carotene, it was concluded that the preparation contained the all-*trans* isomer only. The identity of tracer labelled [11,12-^{3}H]-β-carotene was confirmed from the mass spectrum of the HPLC purified product (m/z 536). The specific activity of 3.8×10^{8} Bq/mmol is consistent with synthesis of the desired product.

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