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SYNTHESIS OF TRITIUM LABELLED 20-HYDROXY-HEPOXILIN A_3, THE MAJOR HEPOXILIN METABOLITE IN HUMAN NEUTROPHILS

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SUMMARY

Methyl (8S)-[20-^{3}H]-8,20-dihydroxy-11,12-epoxy-5Z,9E,14Z-eicosatrienoate ((8S)-[20-^{3}H]-20-hydroxy-HxA_3 methyl ester), a radioactive analog of the HxA_3 metabolite was prepared. The synthesis of the 20-hydroxy analog of HxA_3 methyl ester was performed by the reduction of the corresponding 20-aldehydo-HxA_3 with sodium [^{3}H]-borohydride. The purified [20-^{3}H]-20-hydroxy-HxA_3 methyl ester had a specific activity of 2.5-2.7 Ci/mmol.

Key Words: eicosanoids, hepoxilin A_3, 20-hydroxy-hepoxilin A_3, sodium [^{3}H]-borohydride, tritiation, anthryl esters.

INTRODUCTION

We have recently shown that intact human neutrophils selectively metabolize hepoxilin A_3 (HxA_3) into its 20-hydroxy metabolite [1]. This metabolite was characterized through total chemical synthesis [2]. The metabolite is of interest because it initiates biological action leading to a rise in intracellular calcium [1] and offers an entry into the characterization of the hepoxilin receptor [3]. In this paper we demonstrate that this metabolite can be radiolabelled for further studies on its specific binding to intracellular compartments.

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Sodium borotritiide is a useful reagent for labelling, allowing the introduction of a tritium label into a number of biologically active compounds via their aldehyde or oxo precursors [4]. This method represents a simple and practical preparative route to labelled compounds of biological interest containing a relatively high specific activity. The application of sodium borotritiide was recently described, in particular, for the labelling of carbohydrates [5] and phospholipids [6]. In the present article, we report the synthesis of tritium-labelled ω-hydroxy-hepoxilin A_3 via its ω-aldehyde, applying the above mentioned procedure.

RESULTS AND DISCUSSION

The synthesis of the 20-oxo-8-O-Bz-(8S)-HxA_3, a key intermediate in the preparation of 20-tritiated HxA_3, was performed on the basis of the synthetic scheme developed for the preparation of 20-hydroxy hepoxilins that we described recently [2]. To distinguish the primary and secondary hydroxyl groups, 20-BDPS-protected 20-hydroxy-(10R)-hepoxilin B_3 (**1**), obtained by selective hydrogenation of its triacetylenic precursor [2], was used. According to the presented sequence, compound (**1**) was converted into the benzoate (**2a**) using a known procedure [7]. It is known that the Mitsunobu reaction of secondary allylic *syn*-α,β-epoxy alcohols such as (**1**), is accompanied by the partial allylic rearrangement that leads, in our case, to the 20-BDPSO-hepoxilins B_3 allylic isomer, namely 20-BDPSO-hepoxilin A_3 8-benzoate (**2a**) with the R-configuration of the 8-hydroxyl group. The stereochemical features of this reaction have been reported [8]. Benzoate (**2a**) was worked up with K_2CO_3 in methanol giving allylic (8R)-hydroxy epoxide (**2b**). The Mitsunobu reaction was repeated for the hydroxy epoxide (**2b**) to obtain, with simple inversion in this case, the epoxy benzoate (**3a**), with the desirable 8S-configuration. The hydrolytic stability of the 8-benzoyloxy group allowed the selective removal of the 20-silyl group by treatment with *n*-Bu_4NF giving the primary alcohol (**3b**). Alcohol (**3b**) was smoothly oxidized into the aldehyde (**4a**) using pyridinium dichromate. The benzoate group in (**4a**) was treated with K_2CO_3 leading to the key compound, 20-oxo-(8S)-HxA_3 (**4b**). A 500 MHz ^1H NMR spectrum of the aldehyde (**4b**) clearly supported the structure. Among others, it contains diagnostic signals of an allylic carbinolic H^8 at δ 4.22 ppm as a broad multiplet and of an aldehyde proton as a sharp triplet at δ 9.77 ppm. A corresponding signal for H^{19} was observed at δ 2.44 ppm having a coupling constant of 1.5 Hz (see Experimental).

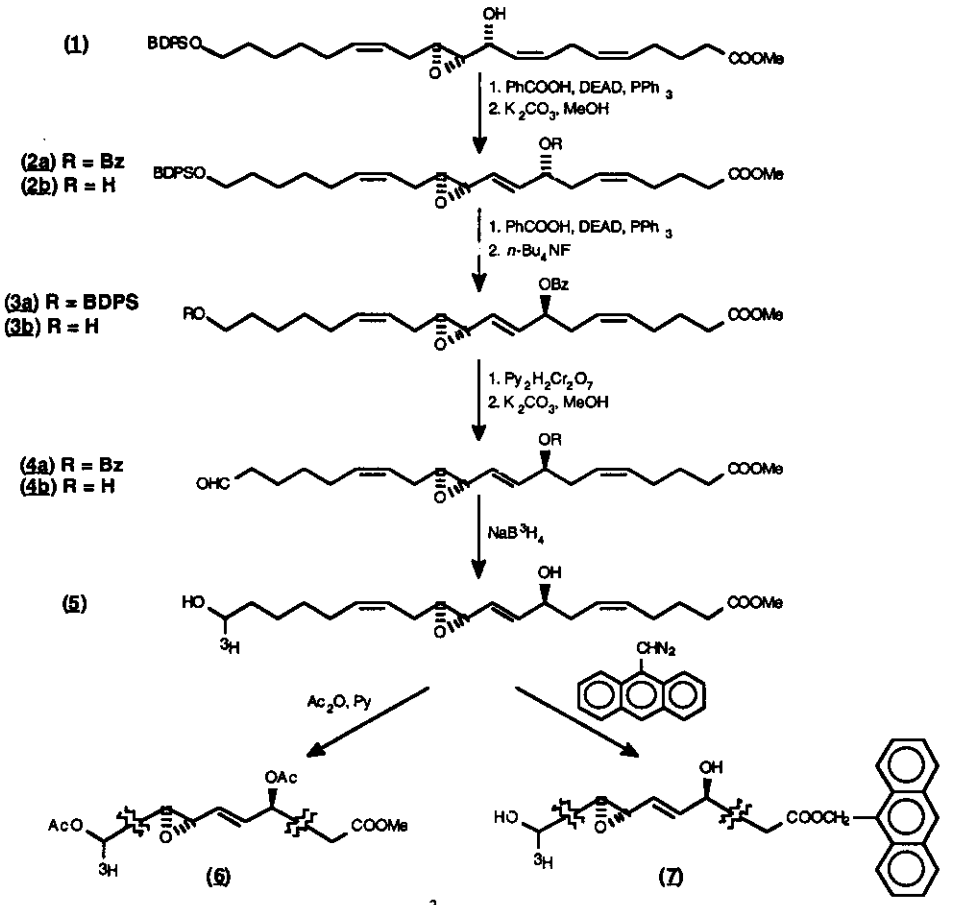
Aldehyde reduction of (**4b**) was carried out with a 0.1N NaOH solution of sodium [^3H]-borohydride mixed with an equal volume of phosphate buffer acidified with HCl to pH 4. The final pH of the reaction mixture was 9 to avoid possible hydrolysis of the methyl ester and expected side reactions of the aldehyde group in basic conditions. Sodium [^3H]-borohydride was used in a 10-fold lesser amount than the aldehyde to secure a high level of the tritium inclusion in the product. The

labelled 20-hydroxy-HxA_3 was purified using TLC. The resulting tritium labelled 20-hydroxy-HxA_3 methyl ester (**5**) as well as its *bis*-acetate (**6**) exhibited the same chromatographic properties as their protium analogs obtained by total chemical synthesis [2]. The specific activity was determined to be 2.5-2.7 Ci/mmol by alkali hydrolysis of the methyl ester followed by treatment with anthryldiazomethane (ADAM) leading to the anthryl ester (**7**). Use of this fluorescent derivative permits detection of the compound in picomolar concentrations [9,10] for quantitative analysis employing the methyl ester of anthracene-9-carboxylic acid as an internal standard (Figure 1). The radiochemical yield of the product was approximately 31%. The range of radiochemical yields obtained in two previous studies was 8 and 44%, the former with carbohydrate aldehydes, the latter with phospholipids [5,6].

EXPERIMENTAL

Materials and Methods

Compound (**1**) was obtained as described previously [2]. Sodium [^{3}H]-borohydride (specific activity 20 Ci/mmol) was purchased from Amersham Canada Ltd. (Oakville, ON). All experimental conditions were optimized using non-radioactive materials. Labelled 20-hydroxy-HxA_3 methyl ester (**5**) was converted into its anthryl ester (**7**) as described [10]. 9-Anthryldiazomethane (ADAM) was purchased from Research Organics Inc. (Cleveland, OH). Methyl ester of anthracene-9-carboxylic acid used as an internal standard was obtained by the treatment of commercially available anthracene-9-carboxylic acid (Aldrich, Milwaukee, WI) with diazomethane. The identity of the products (**6**) and (**7**) was confirmed by a chromatographic comparison with authentic unlabelled compounds. Precoated thin layer chromatographic silica gel 60 plates, layer thickness 250 μm, were obtained from Merck (Darmstadt, Germany). For radioactivity detection on TLC, a Berthold LB 2722 TLC radioactivity scanner was used. Liquid scintillation counting was performed on a Beckman LS 3800 counter using EcoLite as a liquid scintillation cocktail. For sample purification, small columns (5 × 40 mm) were packed with silica gel 63-200 μm (Caledon, Georgetown, ON). High performance liquid chromatography was performed on a 6000 series pump with an LC 480 UV detector (Waters/Millipore, Bedford, MA) at λ 205 nm for the compounds (**2a**), (**3a,b**), (**4b**); a Kratos FS 950 fluorescence detector was used for the compound (**7**). A μPorasil normal phase column (7.8 × 300 mm and 3.9 × 300 mm - Waters/Millipore) was used for the analysis and separation of compounds (**2b**), (**3a**) (**7**), and a Nova-Pak reverse phase column (3.9 × 300 mm - Waters/Millipore) was used for the analysis and separation of compounds (**3b**), (**4b**) (see Experimental details below). Radioactivity in HPLC effluent was carried out by collecting fractions and LS-counting of aliquots (10%) of each fraction. Benzoic acid and diethyl azodicarboxylate (DEAD) were purchased from Sigma (St. Louis, MO) and triphenylphosphine (PPh_3) was from Aldrich (Milwaukee, WI).



Scheme 1. Synthesis of (8S)-[20-^{3}H]-20-hydroxy-HxA$_3$ methyl ester (5).

Methyl ester of 20-*t*-butyldiphenylsilyloxy-(8R)-HxA$_3$ (2b). To a solution of 20 mg (33 μmol) of (10R)-epimer of 20-*t*-BDPSO-HxB$_3$ methyl ester (1) in 1.0 ml of benzene, 16.2 mg (132 μmol) of benzoic acid, 23.0 mg (132 μmol) of DEAD, and 34.8 mg (132 μmol) of PPh_3 were added successively at 20°C. The reaction mixture was stirred until the yellow color had disappeared (2 min). The solvent was taken to dryness and the mixture of products containing benzoate (2a) was treated with K_2CO_3 in methanol for 5 h at 20°C and then passed through a column of silica gel. The mixture containing the HxA$_3$ derivative (2b) was purified by SP-HPLC on a 7.8 × 300 mm column (0.7% *i*-PrOH in hexane, 7.0 ml/min, retention time 24.2 min) giving 7.6 mg of (2b) (yield 38%; R_f 0.35; EtOAc-hexane, 2:3).

Methyl ester of 20-hydroxy-8-O-benzoyl-(8S)-HxA$_3$ (3b). 7.6 mg (12.5 μmol) of 20-*t*-BDPSO-(8R)-HxA$_3$ (2b) was converted into the 20-*t*-BDPSO-(8S)-benzoate (3a) in the same way as described above. The benzoate (3a) was purified by SP-HPLC on a 7.8 × 300 mm column (0.3% *i*-PrOH in hexane, 5.0 ml/min, retention

time 13.4 min, yield 8.1 mg, 91% (R_f 0.61; EtOAc-hexane, 2:3)), and dissolved in 1 ml of THF. 100 µl of 1.0 M solution of *n*-Bu_4NF in THF was added at 20°C. After 3 h of stirring, 5 ml of water and 1 ml of EtOAc were added. The organic phase was separated, taken to dryness, re-dissolved in benzene and filtered through a silica gel column 5 × 40 mm, eluent EtOAc-hexane, 1:1 (10 ml) giving 5.1 mg (87% from **2b**) of the 20-hydroxy-alcohol (**2**), (R_f 0.16; EtOAc-hexane, 2:3). RP-HPLC gave a single peak at 7.4 min (MeCN-H_2O, 70:30, flow rate 1.0 ml/min).

Methyl ester of 20-oxo-(8S)-HxA_3 (**4b**). A suspension of pyridinium dichromate (6.2 mg, 16.5 µmol) and freshly activated grounded molecular sieves 3Å(5 mg) in 0.7 ml of dichloromethane were added to a solution of the alcohol (**3b**) (4.0 mg, 8.5 µmol) in 0.3 ml of dichloromethane. The mixture was stirred for 1 h at 20°C, then quenched with 10 µl of methanol and passed through a silica gel column 5 × 40 mm, and eluted with CH_2Cl_2 (10 ml) → EtOAc-hexane, 1:1 (10 ml). After evaporation of the solvent, 3.7 mg (92%) of aldehydobenzoate (**4a**) was obtained, (R_f 0.45; EtOAc-hexane, 2:3). To a solution of the aldehydobenzoate (**4a**) (3.7 mg, 7.9 µmol) in 0.5 ml of MeOH, a fine suspension of K_2CO_3 (5 mg, 36 µmol) was added. After 4 h of stirring 1 ml of phosphate buffer (pH 6) was added, and the reaction mixture was extracted with 3 × 0.5 ml of EtOAc and taken to dryness. The final purification was performed on a RP-HPLC (MeCN-H_2O, 60:40, flow rate 0.5 ml/min, retention time 9.7 min), leading to 2.7 mg (86% from **3b**) of the hydroxyaldehyde (**4b**), (R_f 0.16; EtOAc-Hx, 2:3). NMR (500 MHz, $CDCl_3$, δ, ppm): 1.41 and 1.64 (2 × m, 4H, H^{17} + H^{18}), 1.71 (quintet, 2H, J 7.5 Hz, H^3), 2.05-2.12 (m, 4H, H^4 + H^{16}), 2.32 (t, 2H, J 7.5 Hz, H^2), 2.32 and 2.38 (2 × m, 4H, H^7 + H^{13}), 2.44 (dt, 2H, J 1.5 and 7.0 Hz, H^{19}), 2.88 (dt, 1H, J 1.8 and 5.0 Hz, H^{12}), 3.16 (dd, 1H, J 1.8 and 7.8 Hz, H^{11}), 3.67 (s, 3H, OMe), 4.22 (m, 1H, H^8), 5.40-5.57 (m, 5H, olefinic H), 5.95 (dd, 1H, J 5.7 and 15.2 Hz, H^9), 9.77 (t, 1H, J 1.5 Hz, CHO).

Methyl (8S)-[20-^{3}H]-8,20-dihydroxy-11,12-epoxy-5Z,9E,14Z-eicosatrienoate (8S)-[20-^{3}H]-20-hydroxy-HxA_3 methyl ester) (**5**). To a solution of 2 µg (5.5 nmol) aldehyde (**4b**) in 50 µl MeOH and 50 µl 0.1M phosphate buffer acidified to pH 4.0 with 0.1 M HCl, a solution of sodium [^{3}H]-borohydride (specific activity 20 Ci/mmol, 10 µCi, 0.5 nmol) in 0.1 M NaOH was added. The mixture was allowed to stay at 20°C for 2 h. The whole mixture was loaded onto a TLC plate and developed with 100% EtOAc. The zone with R_f 0.33 was scraped off, washed with 5% MeOH in EtOAc and the solvent was taken to dryness. Yield of labelled 20-hydroxy-HxA_3 methyl ester (**5**) was 3.1 µCi, approx. 1.2 nmol (calculated from specific activity, see later - radiochemical yield 31%).

Methyl ester of [20-^{3}H]-8,20-*bis*-acetoxy-(8S)-HxA_3 (**6**). To 0.05 µCi of labelled HxA_3 methyl ester (**5**), 10 µl of pyridine and 10 µl of acetic anhydride were added consequently. The mixture was allowed to stay at room temperature for 12 h.

Reagents were taken to dryness, the residue was re-dissolved in benzene and loaded onto a TLC plate, eluent EtOAc ethyl acetate-hexane, 3:2. The bis-acetate exhibited R_f 0.56, whereas starting diol had R_f 0.14. The TLC properties were essentially the same as for the non-labelled standards, obtained by total synthesis [2].

Determination of the specific activity of (8S)-[20-^{3}H]-20-hydroxy-HxA_3 methyl ester (5). 0.33 μCi of labelled HxA_3 methyl ester (5) was trans-esterified into its anthryl ester (7) by saponification with 1:1 mixture of MeOH and aqueous solution of 3% NaOH (2 h, 20°C) followed by acidification with 1% HCl to pH 4-5, extraction with diethyl ether (2 x 0.5 ml) and addition of ADAM (100 μg, 0.46 μmol in 100 μl diethyl ether) for 2 h at 20°C giving after evaporation of the solvent 0.30 μCi of the crude ADAM-ester (7) (90%). The fluorescent ester (7) was purified on TLC (EtOAc-Hx, 3:2, 2 developments, R_f 0.35 whereas R_f of the methyl ester (5) is 0.27) and on a 3.9 × 300 mm μPorasil column, (EtOAc-hexane, 1:1, flow rate 2.0 ml/min, fluorescence detection at $\lambda_{exc} \geq 254$ nm, $\lambda_{em} \geq 400$ nm). A comparative analysis was performed with the methyl ester of anthracene-9-carboxylic acid as an internal standard using a reverse phase HPLC (a Nova-Pak column - Waters/Millipore, 3.9 ×

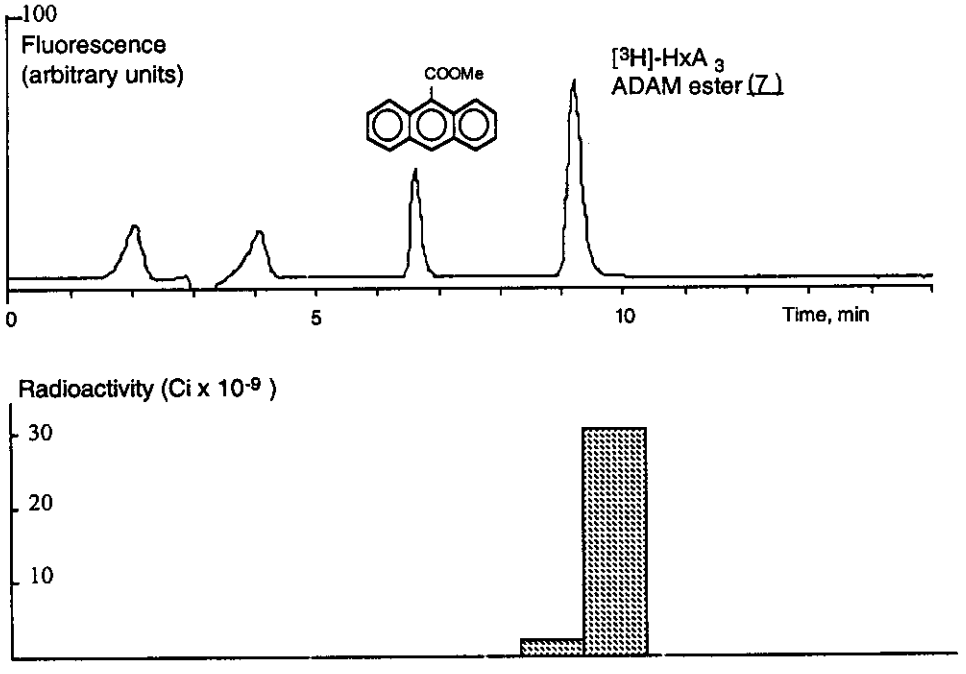


Fig. 1. Comparative HPLC analysis of anthryl ester of (8S)-[20-^{3}H]-20-hydroxy-HxA_3 (7) and 5.0 pmol of anthracene-9-carboxylic acid methyl ester. The top chromatogram was obtained with a fluorescent detector. The lower radiochromatogram was obtained by collecting 1-min fractions of the effluent and counting each fraction with an LS-counter.

300 mm, MeCN-H_2O, 70:30, flow rate 1.0 ml/min, fluorescence detection at $\lambda_{exc} \geq$ 254 nm, $\lambda_{em} \geq 400$ nm); 5-10 pmol of the material was injected (Figure 1). Under these conditions the retention time for (8S)-[20-^{3}H]-20-hydroxy-HxA_3 anthryl ester (7) was 9.1 min, whereas for methyl ester of anthracene-9-carboxylic acid it was 6.7 min. A standard curve comparing responses to different amounts of anthracene-9-carboxylic acid methyl ester was generated and the relative response of 2 different concentrations of [20-^{3}H]-HxA_3 methyl ester (5) was evaluated. On this basis the specific activity was determined to be 2.5-2.7 Ci/mmol.

CONCLUSION

We describe herein a simple and efficient method for the preparation of tritium labelled terminally oxidized hepoxilins through sodium borotritiide reduction. This method may be useful for other lipids containing oxo and/or aldehyde groups for the generation of appropriate radioactive tools of high specific radioactivity for biological and receptor binding investigations.

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