Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXXIV, No. 3

SYNTHESIS OF METHYL [5,6,8,9,14,15-$^3H_6$]-HEPOXILIN B_3 AND ITS CONVERSION INTO METHYL [5,6,8,9,14,15-$^3H_6$]-HEPOXILIN A_3

P.M.Demin[1,2], K.K.Pivnitsky[2], L.L.Vasiljeva[2], and C.R.Pace-Asciak[1,3,§]

[1]*Research Institute, Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8;*

[2]*Institute of Experimental Endocrinology, National Endocrinology Scientific Centre RAMS, Moscow 115522, Russian Federation;*

[3]*Department of Pharmacology, University of Toronto, Ontario, Canada M5S 1A8*

SUMMARY

The selective tritiation of methyl 10(R/S)-hydroxy-11(R),12(S)-epoxyeicosa-5,8,14-triynoate into two C^{10}-epimeric [$^3H_6$]-hepoxilins B_3 methyl esters is described. These compounds are subsequently converted into two C^8-epimeric [$^3H_6$]-hepoxilins A_3 methyl esters by allylic rearrangement under Mitsunobu conditions via corresponding benzoates.

Key words: hepoxilin A_3, hepoxilin B_3, tritiation, Mitsunobu inversion, allylic rearrangement

INTRODUCTION

Hepoxilins A_3 and B_3 (HxA_3 and HxB_3) are biologically active products of the 12-lipoxygenase pathway formed through the rearrangement of 12(S)-hydroperoxyeicosatetraenoic acid (1). Hepoxilins have been shown to stimulate insulin secretion (2), to enhance Ca^{2+} transport across membranes (3), to mobilize intracellular calcium stores (4), to potentiate hormone-induced vascular permeability (5) and contraction (6,7). Hepoxilins have pre-synaptic actions in the mammalian (8,9) and *Aplysia* CNS (10). Recently hepoxilins have been identified as endogenous lipids mediating volume regulation in human platelets (11). To investigate the metabolism of these important native compounds both *in vitro* and *in vivo*, availability of labelled analogs with high molar specific radioactivity as suitable instruments are needed.

§To whom correspondence should be addressed.

CCC 0362–4803/94/030221–10

Received 5 August, 1993
Revised 21 October, 1993

RESULTS AND DISCUSSION

The widely accepted approach to obtain deuterium- and tritium-labelled eicosanoids is through use of their acetylenic precursors. This permits the introduction of hydrogen isotopes into the molecule (12). Hepoxilins of high specific radioactivity are currently unavailable. In this connection and having developed synthetic methods to prepare the appropriate acetylenic precursor of hepoxilins, we set out to develop appropriate techniques for carrying out and optimizing its hydrogenation/tritiation. It is known that the hydrogenation of triple bonds in such unstable compounds is a difficult process and its selectivity varies with the nature of substrate. In most cases using Lindlar catalyst, the deactivating amines, such as quinoline or pyridine must also be added, especially when multifunctional polyacetylenes are used (13-17); the tritiation of the polyacetylenic precursors of dihomo-γ-linolenic and arachidonic acids over Lindlar catalyst not requiring the deactivating amines has been described (18).

Our approach to the synthesis of tritium-labelled hepoxilins is based on the triacetylenic analog of HxB_3, i.e. methyl 10(R/S)-hydroxy-11(R),12(S)-epoxy-5,8,14-eicosatriynoate (1), obtained by total synthesis (19). Previously we had developed the selective (≥95%) (19,20) hydrogenation of the acetylene (1) into HxB_3 methyl esters on Lindlar catalyst. This method employs equal amounts of catalyst (1:1 w/w) in the presence of quinoline and repeated removal/addition of catalyst until hydrogenation is complete due to the lability of the triacetylene and accompanied poisoning of the catalyst under these conditions. It was shown under these conditions that hydrogenation with the first addition of Lindlar catalyst leads to minor formation of the desirable triene (≤5%) with 95% residual triene, while in the absence of quinoline using any amount of catalyst, over hydrogenated products are preferred.

The above general method of hydrogenation could not be applied to tritiation experiments since replacement of catalyst several times after exposure to 100% tritium gas is not practical. Hence the procedure needed modification to ensure the tritiation of triple bonds with maximum selectivity with a single addition of catalyst. We therefore developed a modified procedure of hydrogenation/tritiation using larger amounts of Lindlar catalyst (4:1 w/w) in the presence of quinoline; an excess of catalyst was used to prevent its complete inactivation during the reaction. These conditions seem to be optimal. This method improves the yield of desirable triene in the reaction mixture, and over-reduced material is observed as major by-products in model experiments*. Components of the reaction mixture were identified by GCMS of the TBDMSi derivatives (see Experimental section). The complex mixture containing the two epimeric $[^3H_6]$-HxB_3 (2a,b) and over tritiated products, was separated by argentation TLC** (Fig. 1A) to afford a purified mixture of the two epimers of $[^3H_6]$-HxB_3 (Fig. 1B). The separation of $[^3H]$-labeled prostaglandins with different degrees of saturation on $AgNO_3$-impregnated TLC plates has been described (21,22). Subsequently, the $[^3H_6]$-HxB_3 epimers (theoretical spec. act. 169 Ci/mmol) were easily separated from each other using normal phase HPLC. The ratio between the two C^{10} -epimers obtained was 65:35 with a preference for $[^3H_6]$-(10R)-HxB_3 (2a) as in the starting triacetylene epimeric mixture (1) (19). The radiochemical purity of each epimer was >97% (Fig. 2A, B).

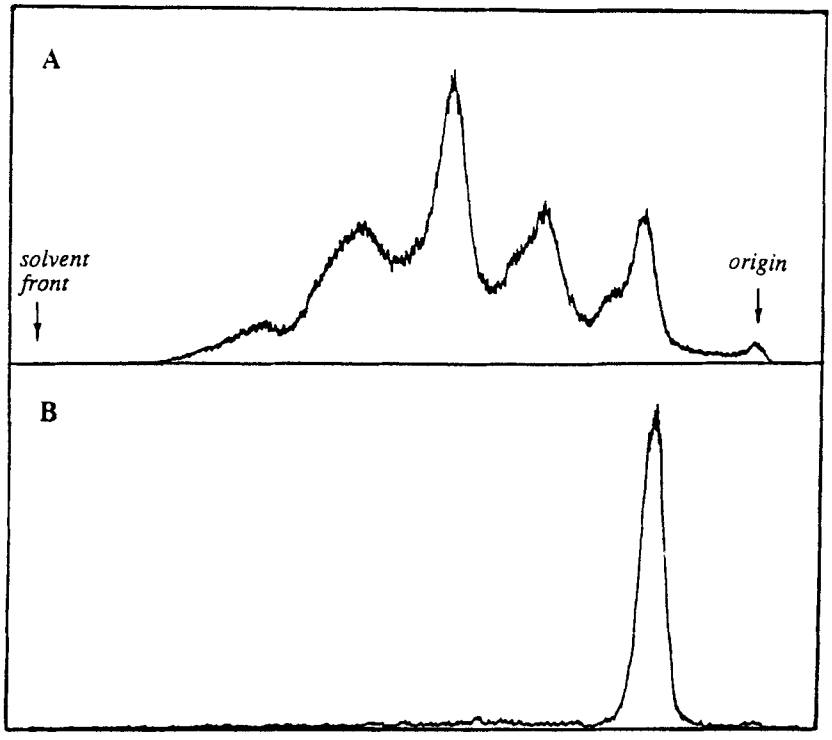

Fig. 1. $AgNO_3$-impregnated thin-layer radiochromatograms of the crude tritiation products (**A**) and [^{3}H]-HxB_3 methyl esters (2a,b) after purification (**B**). The solvent was ethyl acetate-methanol-acetic acid 100:0.50:0.25 (v/v/v).

We previously described a convenient way to convert HxB_3 into HxA_3 through allylic rearrangement under appropriate conditions (23). This involved the formation of HxB_3 mesylates, and their hydrolysis with rearrangement to form the corresponding HxA_3. However, this procedure is not efficient when small amounts of HxB_3 are employed as in the case of the tritiation products where < 1μg of HxB_3 is handled due to the high specific activity of these compounds. Under those conditions an excess of reagents led mainly to the formation of radioactive dehydration products (>60%).

To obtain the corresponding [$^3H_6$]-HxA_3 (3a,b) from [$^3H_6$]-HxB_3 (2a,b) in good yield, we employed a new method of allylic rearrangement based on the Mitsunobu inversion (24). The allylic transposition accompanying the Mitsunobu reaction is a previously described reaction for some allylic stable secondary alcohols (25, 26) although it has not been described for the epoxy alcohols of the type used in this study. We established that the workup of [$^3H_6$]-HxB_3 (2a,b) under Mitsunobu conditions (diethylazodicarboxylate (DEAD) / triphenylphosphine / benzoic acid in THF at 20°C) (24) resulted in a mixture of [$^3H_6$]-HxA_3 and [$^3H_6$]-HxB_3 benzoates followed by mild treatment with MeONa in MeOH to hydrolyze the benzoates. This one-pot reaction system minimizes handling of mCi amounts of radioactive products and permits the use of an excess of reagents without detectable decomposition of the reaction products.

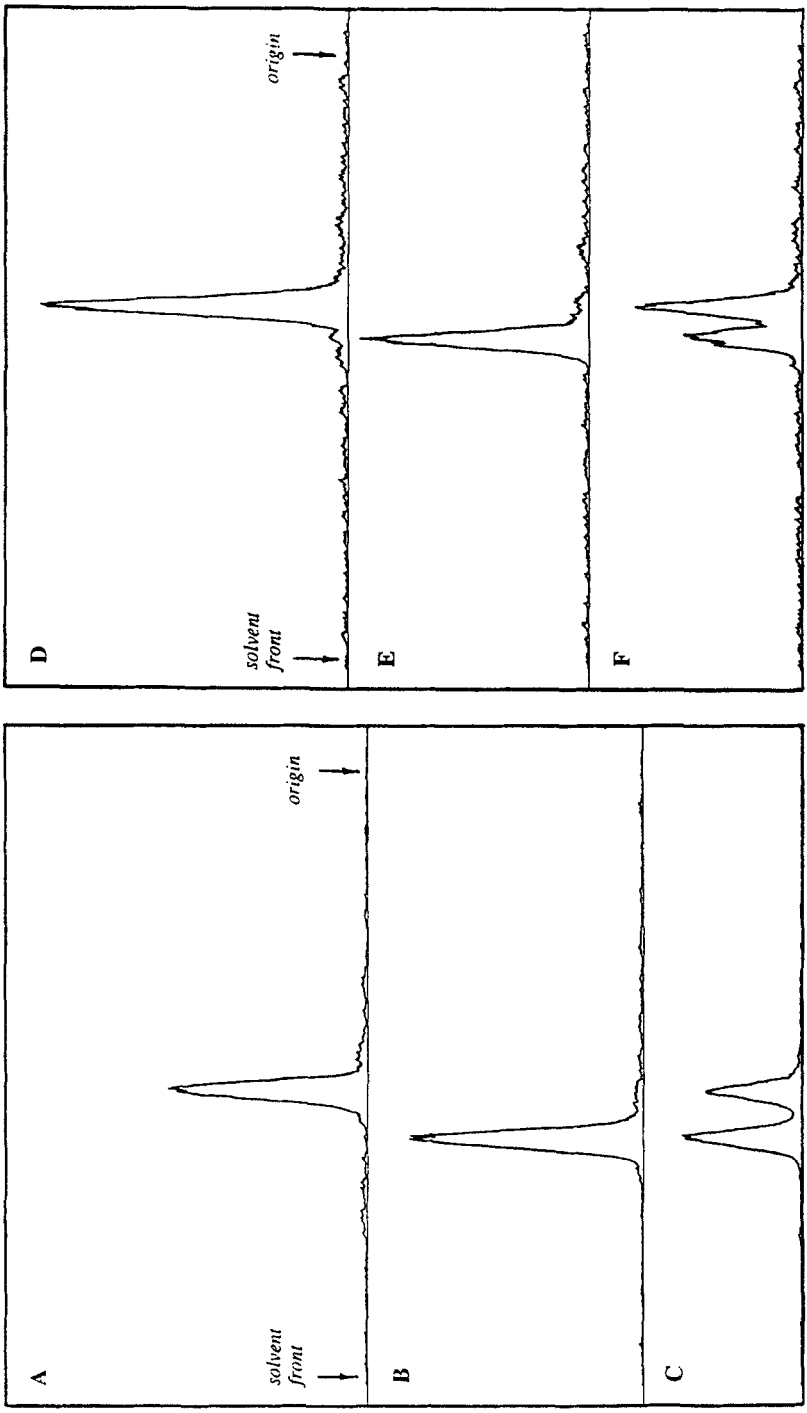

Fig. 2. Thin-layer radiochromatograms of the purified individual [^{3}H]-HxB$_3$ methyl esters (2a) (**A**), (2b) (**B**), and their mixture (**C**); and the purified individual [^{3}H]-HxA$_3$ methyl esters (3a) (**D**), (3b) (**E**), and their mixture (**F**). The system was benzene–diethyl ether 4:1 (v/v), four developments. See Table 1 for R_f values.

For both epimers of [^{3}H]-HxB$_3$ (2a,b), the S_N2- and S_N2^1- type displacements of intermediate complexes (4a,b) by benzoate anion (24) gave an inverted epimer of [$^3H_6$]-HxB$_3$ and the two [$^3H_6$]-HxA$_3$ epimers (3a,b) with preference of the epimer formed by attack of benzoate anion from the side opposite to the leaving group as shown in Fig. 3. Only (10R)-[$^3H_6$]-HxB$_3$ rearranges into [$^3H_6$]-HxA$_3$ epimeric mixture with good yield (up to 41%) affording a 6:1 mixture of (8R)-[$^3H_6$]-HxA$_3$ (3a) and (8S)-[$^3H_6$]-HxA$_3$ (3b) respectively; on the other hand (10S)-[$^3H_6$]-HxB$_3$ gives preferably the (10R)-[$^3H_6$]-HxB$_3$ with only 7% of a 1:6 mixture of (8R)-and (8S)-[$^3H_6$]-HxA$_3$ (3a,b) (Table 1). Thus an actual 65:35 mixture of [$^3H_6$]-(10R) and (10S)-HxB$_3$ (2a,b) is rearranged into the mixture of [$^3H_6$]-(10R) and (10S)-HxB$_3$ (2a,b) and [$^3H_6$]-(8R) and (8S)-HxA$_3$ (3a,b) with a 3:1 ratio between the latter at an overall yield of 28% (Table 1). We could not develop a suitable method for the hepoxilins using a modified Mitsunobu procedure requiring the addition of a catalytic amount of $PdCl_2(MeCN)_2$ to promote the allylic rearrangement (27) since the reactive epoxide in HxA$_3$ could be opened under these conditions.

To reach a better yield of the (8S)-[$^3H_6$]-HxA$_3$ we worked up this mixture once more under the Mitsunobu conditions to invert all (8R)-[$^3H_6$]-HxA$_3$ (3a) into (8S)-[^{3}H]-HxA$_3$ (3b), and to obtain simultaneously an additional quantity of (8R)-[$^3H_6$]-HxA$_3$ (3a) from (10R)-[$^3H_6$]-HxB$_3$ (2a). Under these conditions we obtained a mixture containing two [$^3H_6$]-HxA$_3$ (3a,b) epimers in almost equal ratio (Table 1) which were subsequently separated on a straight-phase HPLC column resulting in pure individual [^{3}H]-HxA$_3$ (3a & 3b) in reasonable yield and good radiochemical purity (Fig 2, D, E).

MATERIALS AND METHODS

Methyl 10-hydroxy-11,12-epoxy-5,8,14-eicosatriynoate (1) was synthesized as described in ref. 19 as an unseparable 65:35 mixture of (10R)- and (10S)-epimers. Lindlar catalyst and quinoline were obtained from Aldrich (USA); diethylazodicarboxylate, benzoic acid and triphenylphosphine were supplied by Sigma (USA). Tetrahydrofuran (THF) was distilled over benzophenoneketyl sodium directly before use. All glassware was silanized with a 1% aq. solution of AquaSil siliconizing fluid (Pierce, USA).

Thin layer chromatography (TLC) was performed on Kieselgel 60 glass-backed silica gel TLC plates (0.25 mm thickness [Merck, FRG]). Ag^+-impregnation of the TLC plates was carried out by dipping the plates in 10% solution of $AgNO_3$ in MeOH-H_2O (5:1, v/v) followed by air drying and activation at 100°C for 30 min. After development with appropriate solvent, the distribution of radioactivity for each sample was determined with a TLC radiochromatogram scanner (Berthold LB 2722 [FRG]). Radioactivity in each sample was measured by a scintillation counter (Beckman LS 3800 [USA]) in EcoLite scintillation liquid (ICN, Canada). High performance liquid chromatography was performed on a Waters system (USA) using a μPorasil column (3.9 x 300 mm) with an on-line flow-through radioactivity monitor (Berthold BF 2240 [FRG]) and PCS scintillation fluid (Amersham, USA) at a flow of 1 ml/min; for the purpose of preparative separation the detector was disconnected and the fractions were analyzed by counting aliquots of effluent to determine the distribution of the radioactivity.

Table I. Results of Mitsunobu inversion of individual or a mixture of epimers of HxB_3 - formation of HxA_3.

Products	Composition (%) of products after Mitsunobu inversion of individual HxB_3 epimers		Composition (%) of products after two sequential Mitsunobu inversions of a 35:65 mixture of (10S)- and (10R)-HxB_3		Chromatographic properties of [^{3}H] hepoxilins		
	(10R)-HxB_3 (2a)	(10S)-HxB_3 (2b)	first inversion	second inversion	Ag^+-TLC R_f[a]	SiO_2-TLC R_f[b]	HPLC retention time, min[c]
(10R)-HxB_3 (2a)	3-4	90-93	36-39	33-39	0.14	0.52	22.9
(10S)-HxB_3 (2b)	54-57	2-3	32-39	18-21	0.14	0.60	20.0
(8R)-HxA_3 (3a)	30-35	1-2	17-23	21-23	not shown	0.42	43.0
(8S)-HxA_3 (3b)	5-6	6-7	6-7	20-23	not shown	0.46	40.9

[a]EtOAc-MeOH-AcOH, 100:0.50:0.25; [b]C_6H_6-Et_2O, 4:1, 3 developments; [c]μPorasil 3.9 x 300 mm, 0.7% i-PrOH in hexane, 1.0 mL/min

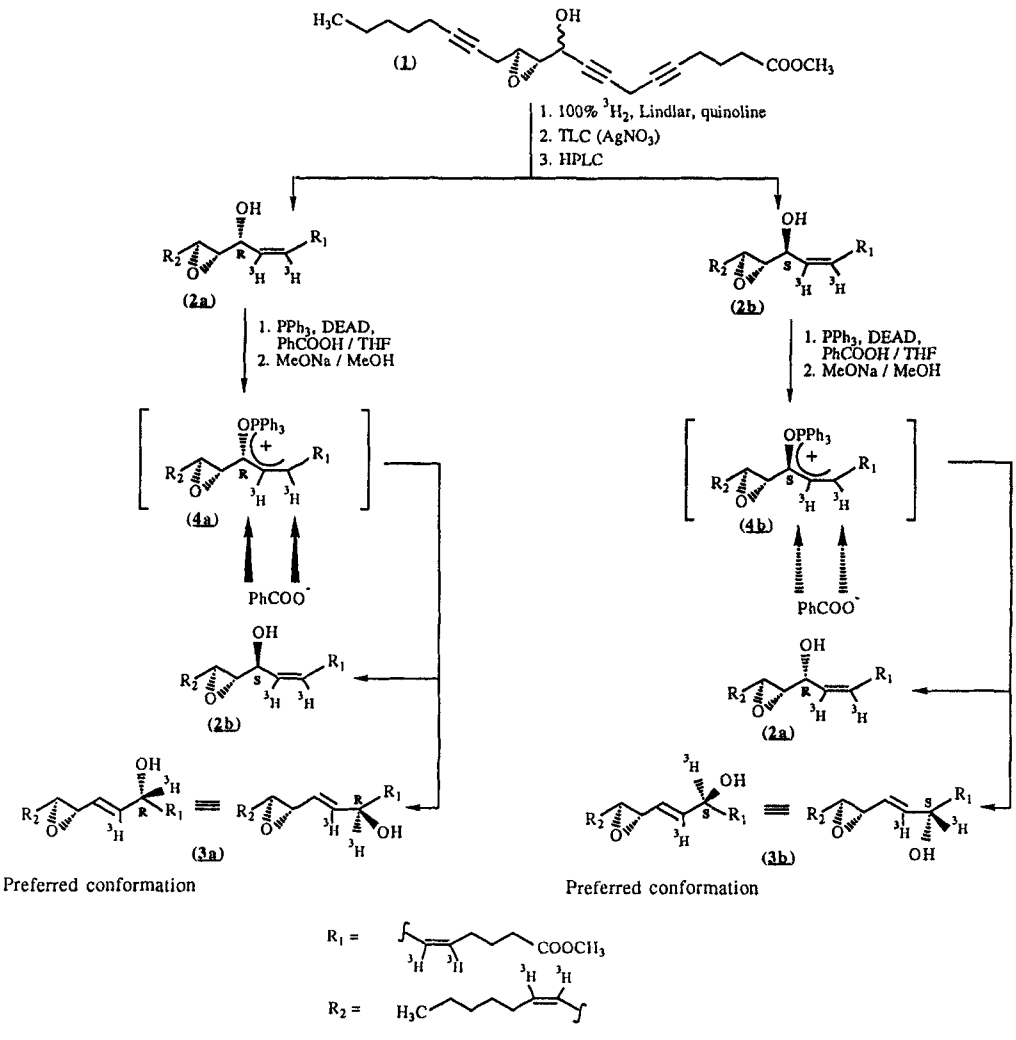

Fig.3. Scheme describing the catalytic tritiation of the acetylenic precursor to form the two epimers of HxB_3 (2a,b) which were isolated and individually converted through the Mitsunobu reaction to HxA_3 (3a,b). In this process the inverted epimers of HxB_3 were also isolated indicating attack of the benzoate anion from the *opposite* side of the Ph_3PO- leaving group. The preferred conformation of HxA_3 is indicated.

In model experiments investigating hydrogenation of the triacetylenic precursor, the reaction mixtures were converted into their TBDMSi derivatives using t-butyldimethylsilylimidazole-dimethylformamide kit (Supelco, USA), heating at 65°C for 5 min, extracting the derivative with hexane and analyzing aliquots of the hexane extract on a gas chromatograph (Hewlett-Packard 5700A) with a glass capillary column SPB-1 (60 m x 0.3 mm) with on-column injection. Helium was used as carrier gas. The same samples were subsequently analyzed on a gas chromatograph-mass spectrometer (Hewlett-Packard 5988A) equipped with an OV-1 fused silica capillary column (12 m x 0.2 mm), an on column injector and hydrogen as carrier gas.

As standards, epimeric HxA_3 and HxB_3 methyl esters obtained by synthesis (19,23) and appropriately derivatized were used.

[5,6,8,9,14,15-$^3H_6$]-HxB_3 methyl esters (2a,b)

A. Model experiment

A mixture of triacetylenic precursor (1) (1.0 mg), Lindlar catalyst (4.0 mg), and quinoline (2.5 µl) in dry benzene was vigorously stirred in a hydrogen atmosphere (1 ATM) at 20°C until hydrogen absorption had ceased (40 min, total hydrogen absorbed 0.8 mL). The catalyst was filtered off and the filtrate was analyzed on $AgNO_3$-impregnated silica gel TLC plate (EtOAc-MeOH-AcOH, 100:0.50:0.25, v/v/v). The desired triene (2) had R_f = 0.14, while the over-hydrogenated by-products showed R_f = 0.45 and 0.36. GC-MS analysis of their tBDMSi derivatives showed the reaction mixture to contain 41% of HxB_3 (prominent ion m/e 407 [M - tBu]$^+$ and the balance made up of over hydrogenated products (m/e 409). Both TLC on normal silica gel and straight phase HPLC analysis showed no difference in migration between epimeric HxB_3 and by-products. Hence Ag^+-impregnated TLC was essential in separating the desired compounds.

B. Tritiation and preparative separation of [$^3H_6$]-HxB_3 (2a,b)

Tritiation was carried out at Dupont-NEN (Boston, MA) under the supervision of Dr. Dave Ahearn, essentially under the same conditions as in the model experiment above except that tritium gas (1.5 ATM) was employed. After tritiation, the catalyst was filtered and the solvent concentrated in vacuo. Exchangeable tritium was removed with isopropanol/triethylamine (99/1, v/v) and the solvent was evaporated in vacuo. The total radioactivity in the residue was 200 mCi (40% of the theoretical amount). This residue was purified on preparative silica gel TLC plates (20 x 20 cm) impregnated with 10% $AgNO_3$. The plates were developed with EtOAc-MeOH-AcOH (100:0.50:0.25, v/v/v) and the desired zone (R_f 0.12-0.18) was identified by radioscanning (Fig. 1A). The appropriate zone of silica gel (R_f 0.12-0.18) was scraped into a test tube and the material was eluted from the adsorbent with EtOAc (50 mL). A second TLC was required to obtain >95% pure HxB_3 as a mixture of two epimers. The isolated yield of [$^3H_6$]-HxB_3 (2a,b) obtained was 1.7% (8.1 mCi). Subsequent HPLC analysis (Table 1) showed that the isolated product was made up of a mixture of two well resolved epimers with a ratio of 65:35 between (10R)-, and (10S)-[$^3H_6$]-HxB_3 (2a), (2b), which were preparatively separated using the same HPLC conditions (see Table 1).

[5,6,8,9,14,15-^3H]-HxA_3 methyl esters (3a,b)

To a solution of a mixture of (10R)- and (10S)-epimers (65:35 ratio) of [$^3H_6$]-HxB_3 (2a,b) (400 µCi) in 300 µl of dry THF, PPh_3, PhCOOH and DEAD (10 nM ea) were added successively at 20°C. After 10 min, the reaction mixture was analyzed on TLC showing the complete conversion into corresponding benzoates (R_f 0.56, EtOAc-hexane, 3:7). The reaction was quenched by addition of 100 µl of MeOH, and the solvent was evaporated to dryness. The benzoate group was removed by the addition of 25% MeONa in MeOH (200 µl, 2 h, 20°C). The reaction mixture was separated from the reagents on 20 x 20 cm TLC plate with silica gel and EtOAc-hexane (3:7 v/v) as developing solvent. The radioactive zone was removed and washed with 50 mL of EtOAc. HPLC analysis on normal phase showed the mixture of [$^3H_6$]- (10R)-, and (10S)-HxB_3 (2a,b) and [$^3H_6$]- (8R)-, and (8S)-HxA_3 (3a,b) (Table 1). The above described procedure was repeated with the entire

mixture of [$^3H_6$]-Hx to increase the yield of (8S)-[$^3H_6$]-HxA_3 (3b) followed by HPLC analysis (Table 1). The separation of the two [$^3H_6$]-HxA_3 epimers (3a,b) on HPLC in the same conditions led to individual [$^3H_6$]-HxA_3 (3a), (3b). The isolated yield of each epimer from HxB_3 after two steps was approx. 15% (60 µCi).

ACKNOWLEDGEMENTS

This study was supported by grants to CRP-A from the MRC of Canada (MT-4181) and from ZymoGenetics. We thank Dr. Dave Ahearn (Dupont-NEN, Boston, MA) for generously carrying out the tritiation experiment.

REFERENCES

1. Pace-Asciak C.R., Granstrom E., and Samuelsson B. - J. Biol. Chem. 258: 6835 (1983)
2. Pace-Asciak J.R., and Martin J.M. - Prostaglandins Leukotrienes Med. 16: 173 (1984)
3. Pace-Asciak C.R., Laneuville O., Su W.-G., Corey E.J., Gurevich N., Wu P., and Carlen P.L. - Proc. Natl. Acad. Sci. USA 87: 3037 (1990)
4. Derewlany L.O, Pace-Asciak C.R., and Radde I.C. - Can. J. Physiol. Pharmacol. 62: 1466 (1984)
5. Laneuville O., Corey E.J., Couture R., and Pace-Asciak C.R. - Eicosanoids 4: 95 (1991)
6. Laneuville O., Couture R., and Pace-Asciak C.R. - Br. J. Pharmacol. 105: 297 (1992)
7. Laneuville O., Couture R., and Pace-Asciak C.R. - Br. J. Pharmacol. 107: 808 (1992)
8. Carlen P.L., Gurevich N., Wu P.H., Su W-G., Corey E.J., and Pace-Asciak C.R. - Br. Res. 497: 171 (1989)
9. Pace-Asciak C.R., Laneuville O., Su W-G., Corey E.J., Gurevich N., Wu P., and Carlen P.L. - Proc. Natl. Acad. Sci.USA 87: 3037 (1990)
10. Piomelli D., Shapiro E., Zipkin R., Schwartz J.H., and Feinmark S. - Proc. Natl. Acad. Sci. USA 86: 1721 (1989)
11. Margalit A., Sofer Y., Grossman S., Reynaud D., Pace-Asciak C.R., and Livne, A.- Proc. Natl. Acad. Sci. USA 90: 2589 (1993)
12. Shevchenko V.P., and Myasoedov N.F. - Isot. Phys. Biomed. Sci. 1: 179 (1991)
13. Corey E.J., and Kang J. - J. Amer. Chem. Soc. 103: 4618 (1981)
14. Sprecher H., and Sankarappa K. - Methods Enzymol. 86: 357 (1982)
15. Nicolaou K.C., Veale C.A., Webber S.E., and Katerinopolous H. - J. Amer. Chem. Soc. 107: 7515 (1985)
16. Belosludtsev Yu.Yu., Myagkova G.I., Evstigneeva R.P., Bobrova N.I., and Pivnitsky K.K. - Bioorg. Khim. 13: 1125 (1987), Chem. Abstr. 109: 54511e
17. Lellouche J.P., Aubert F., and Beaucourt J.P. - Tetrahedron Lett. 29: 3069 (1988)
18. Schevchenko V.P., Myagkova G.I., Lazurkina T.Yu., Demin P.M., Shram S.I., Zabolotsky D.A., Nagayev I.Yu, Belosludtsev Yu.Yu., Evstigneeva R.P., and Myasoedov N.F. - J. Labelled Compds. Radiopharm. 27: 1177 (1989)
19. Vasiljeva L.L., Manukina T.A., Demin P.M., Lapitskaya M.A., and Pivnitsky K.K. - Tetrahedron 49: 4099 (1993)

20. Demin P.M., Vasiljeva L.L., Lapitskaya M.A., Belosludtsev Yu.Yu., Myagkova G.I., and Pivnitsky K.K. - Bioorg. Khim. 16: 1125 (1990), Chem. Abstr. 114: 42306g
21. Shevchenko V.P., Nagayev I.Yu., and Myasoedov N.F. - Radiokhimiia 30: 375 (1988), Chem.Abstr. 109: 222622n
22. Shevchenko V.P., Nagayev I.Yu., and Myasoedov N.F. - J. Radioanal. Nuc. Chem. 121: 479 (1988)
23. Demin P.M., Vasiljeva L.L., Myagkova G.I., and Pivnitsky K.K. - Bioorg. Khim. 17: 1133 (1991), Chem. Abstr. 115: 231941s
24. Mitsunobu O. - Synthesis: 1 (1981)
25. Lumin S., Yadagiri P., and Falck J.R. - Tetrahedron Lett. 29: 4237 (1988)
26. Farina V. - Tetrahedron Lett. 30: 6645 (1989)
27. Lumin S., Falck J.R., Capdevila J., and Karara A. - Tetrahedron Lett. 33: 2091 (1992)