Synthesis of Labelled Oxylipins: Leukotriene A₄ and 8-*epi*-Prostaglandin F₂ₐ

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SUMMARY

Tritiated leukotriene A₄ and 8-*epi*-prostaglandin F₂ₐ methyl esters were prepared from the corresponding acetylenic precursors by selective reduction with tritium gas to provide a probe for the metabolite identification of these oxylipins. Partially deactivated Pd-catalysts were prepared from commercial products and their composition was optimized to achieve the better selectivity. A micro-method for the saponification of labelled oxylipins methyl esters was used.

Key words: leukotrienes, isoprostanes, tritium, labelled compounds.

INTRODUCTION

Selective hydrogenation of the corresponding acetylenic precursors with hydrogen isotopes (tritium and deuterium) is a very effective method for preparing labelled biologically active compounds. Acetylenic analogous of some important natural oxylipins[*] have become available as a result of progress in the chemical

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* Oxylipins are compounds originating from polyunsaturated fatty acids by action of enzymes at least on one stage of their formation and encompass prostaglandins, leukotrienes, lipoxines and other related compounds.

syntheses. Thus the syntheses of 14,15-dehydro-LTA$_4$ methyl ester (**I**) and 15(RS)-5,6-dehydro-8-*epi*-PGF$_{2\alpha}$ methyl ester (**II**) have been recently described [1, 2].

It is well known that selective hydrogenation of triple bonds to the corresponding *cis*-double bonds is promoted by some deactivating additives (such as salts of Cd, Sn, Pb, Zn and aromatic amines). Among them lead is reported to be one of the most effective for di-substituted acetylenic functions. The influence of the solvent on hydrogenation selectivity is also an established fact as well [3, 4]. Besides it has been found that the optimum conditions for selective hydrogenation reactions are specific for each class of eicosanoids (polyenoic fatty acids, hepoxylins, prostaglandins, etc.). So, the desired selectivity in the hydrogenation of acetylenic oxylipins [3 - 10] could be achieved by optimizing the composition of the solvent (benzene, acetone, hexane, dioxane or their mixtures were tested), catalyst (to be chosen from a Lindlar-type palladium catalysts) and some deactivating additives.

The purpose of the present study was to synthesize labelled leukotriene A$_4$, 15(R)- and 15(S)-8-*epi*-PGF$_{2\alpha}$ methyl esters of high specific radioactivity and in a good yield, and their acetylenic precursors as well.

RESULTS AND DISCUSSION

14,15-Dehydro-LTA$_4$ methyl ester (**I**) was obtained by coupling of epoxydienal (1 eq) with non-3-ynyl-triphenyl phosphonium iodide (3 eq.) in the presence of *n*-butyllithium (3 eq). The reaction was carried out in a mixture of THF/HMPA at -78°C for 15 min as illustrated in Fig. 1. The yield of 80% was achieved after purification by flash chromatography [2].

The acetylenic methyl ester (**I**) was selectively hydrogenated with gaseous tritium over several catalysts (see Table 1). All our attempts to use known hydrogenation procedures has failed to produce the target product in an acceptable yield. As a rule, either rate of hydrogenation was too slow or large amounts of over-hydrogenated products were formed due to a poor selectivity or complete degradation of the starting acetylenic leukotriene occurred in the presence of aromatic amines (Table 1). In addition it should be noticed that the epoxy moiety of (**I**) was only stable under the basic conditions.
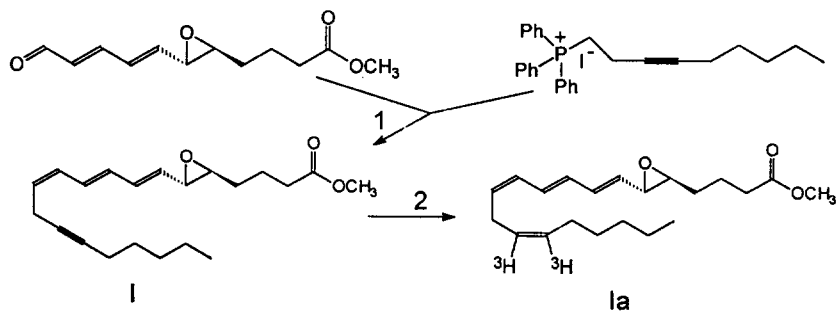
Fig. 1. Synthesis of $[13,14\text{-}^3H_2]$-leukotriene A_4 methyl ester.

Reaction conditions: 1) BuLi/THF/HMPT, -78°C, 15 min;
                        2) $^3H_2$, catalyst (3), HPLC purification.

An acetylenic precursor of methyl arachidonate was used as a model compound for our preliminary experiments because of the ease with wich the reaction could be monitored using chromatography [11]. It was possible to achieve hydrogenation selectivity of up to 70% when 5% Pd/BaSO$_4$ catalyst was modified by using a methanolic solution of lead diacetate (that is similar to that for commercial Lindlar catalyst); a further increase in lead diacetate quantity resulted only in a drop in the hydrogenation rate and increase of the starting acetylenic material in the reaction mixture.

Table 1. Chemical (Sub) and radiochemical (Rad) yields of LTA$_4$ methyl esters in the hydrogenation of its acetylenic precursor with gaseous tritium (amount of acetylenic substrate – 1 mg, reaction time 30 min)

Solvent (0.2 ml)	Catalyst type (and amount, mg)	Additive (and amount, µl)	Sub, %	Rad,%
Hexane-dioxane, 1:1	5% Pd/BaSO$_4$ (5)	NEt$_3$ (2.5)	0.5	0.02
Hexane-dioxane, 1:1	5% Pd/BaSO$_4$ (4)	NEt$_3$ (8)	2	2
Hexane-dioxane, 1:1	Lindlar (6)	–	3	2
Hexane-dioxane, 1:1	Lindlar (4)	NEt$_3$ (2)	5	6
Benzene	Lindlar (6)	NEt$_3$ (12)	7	5
Benzene	Lindlar (6)	Quinoline (3)	8	2
Benzene	Lindlar (5)	Quinoline (7)	10	6
Hexane	Modified Lindlar* (15)	NEt$_3$ (10)	45	40

* Catalyst B (see text below).

More successful proved to be a catalyst, prepared by a consecutive processing of 5% Pd/BaSO$_4$ with methanolic solutions of lead diacetate and sodium

borohydride. According to this technique the yield of methyl arachidonate reached 95% after 15 min and further reaction practically ceased.

The amount of lead diacetate varied from 0 up to a ratio Pd/Pb of 1:2. An optimum Pd/Pb ratio was found to be in the range of 1:0.6 – 1:0.8. The amount of sodium borohydride varied from 0 up to a ratio of 5:1 (a weight ratio 5% Pd/BaSO$_4$/NaBH$_4$ was taken). An optimum value was found for the range 50:1 - 50:4. Further increase of NaBH$_4$ over a ratio of 25:1 did not improve the selectivity of the process but led to a lowering of the rate of hydrogenation. Thus three catalysts were prepared with the Pd/Pb ratio and the catalyst/NaBH$_4$ ratio: 1:0.6 and 50:1(**A**), 1:0.7 and 25:1 (**B**), and 1:0.8 and 25:2 (**C**), correspondingly.

Using the acetylenic analogue of methyl arachidonate as a test compound we found that the mixtures of hexane-dioxane or hexane-NEt$_3$ were the best solvents for hydrogenation (see Table 2).

Table 2. Yield of methyl arachidonate in hydrogenation of its acetylenic analogue by gaseous tritium in the presence of catalyst (B) in different solvents.

Solvent 1, parts	Solvent 2, parts	Yield,%
Hexane, 100	None	73
Hexane, 95	NEt$_3$, 5	80
Hexane, 9	Dioxane, 1	98
Hexane, 4	Dioxane, 1	99
Hexane, 3	Dioxane, 2	98
Hexane, 1	Dioxane, 1	97
Hexane, 3	Dioxane, 7	92
Dioxane, 100	None	75
Chloroform, 100	None	89
Methanol	None	65

In the case of (**I**) the best results were obtained with hexane-NEt$_3$ (95:5) as a solvent and the catalyst (**B**). The conditions led to a target product with 30 – 35% yield (after two stage HPLC purifications) and a specific radioactivity of 40-45 Ci/mmol.

The acetylenic analogue of 15(RS)-8-*epi*-PGF$_{2\alpha}$ (**II**) was prepared according to the synthetic approach, described earlier, via biomimetic pathway of the 5-exo-radical cyclization with molecular oxygen trapping under classical tin hydride

conditions [1] as outlined on Fig. 2. The cyclization was achieved using Bu₃SnH
(1.2 eq) and BEt₃ (1 eq) in dry xylene at room temperature, under a stream of dry
argon, followed by injection of dry air and addition of Ph₃P to afford the protected
isoprostane as a mixture of 15-epimers as a major product of the reaction (yield
55%). The bis-silyl groups were removed later using tetrabutylammonium fluoride
in dry THF at room temperature to give 15(RS)-5,6-dehydro-8-*epi*-PGF$_{2\alpha}$ methyl
ester (II) in 90% yield. This mixture was further used for reduction with tritium and
products were resolved by semi-preparative HPLC into individual labelled 15(S)-
and 15(R)-epimers (IIa and IIb) using corresponding 'cold' 15(S)- and 15(R)-
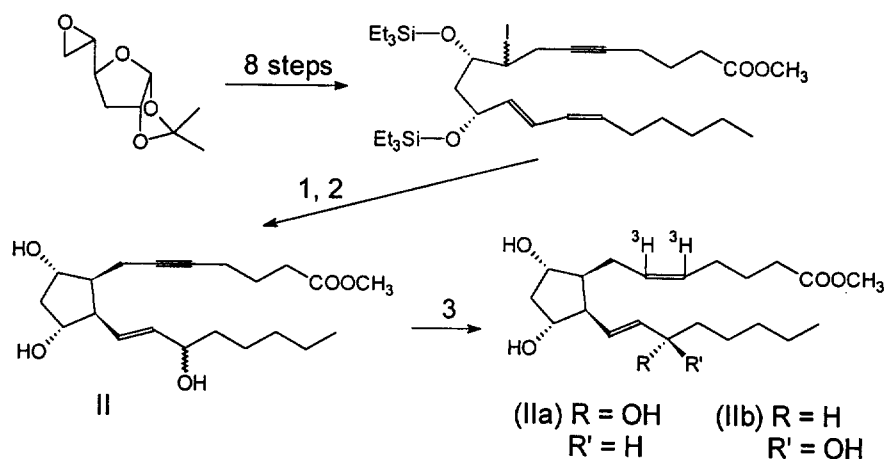epimers as a reference standards.

Fig. 2. Synthesis of [5,6-^{3}H$_2$]-8-*iso*-prostaglandin F$_{2\alpha}$ methyl ester.
Reaction conditions:
1) 1.2 eq of n-Bu₃SnH, 1 eq of BEt₃, O₂, xylene, 20°C, 0.5 hr, 1.1 eq of Ph₃P;
2) 1 eq of n-Bu₄NF, THF, 20°C, 2 hrs;
3) gaseous ^{3}H₂, catalyst B; HPLC purification.

The tritium label was introduced into isoprostane by hydrogenation of ester
(II) over the same catalyst (B) with gaseous tritium in the hexane-dioxane solvent
mixture. The yield of (R,S) epimeric mixture was 28%, and a further 10-15%
increase was observed if NEt₃ was added to the reaction mixture in a catalyst/amine
ratio of 1:5. Under these conditions only 3% of starting compound was detected
after 30 min, while the yield of selectively hydrogenated products of 35-40% and
the specific radioactivity of 50-52 Ci/mmol were reached. It should be mentioned

that under the same reaction conditions but using the Lindlar catalyst [12] a yield of only 16% for labelled iso-prostanes could be obtained.

Using deuterium instead of tritium, this reaction also led to a 35-45% of reduced compounds after 30 min and their structure was confirmed by mass spectrometric analysis (approx. 95% of deuterium inclusion into isoprostane was found).

Different conditions of basic saponification were tested to prepare labelled isoprostanes in free acid form for further biological experiments. The hydrolysis process was monitored by radio-TLC (Table 3). Sodium hydroxide was shown to give the best yield and afforded almost quantitatively 8-*epi*-PGF$_{2\alpha}$ after 4 hours of saponification at room temperature. Simple dilution of the resulting reaction mixture with saline gives a solution of the labelled isoprostane ready for biological experiments.

Table 3. Hydrolysis kinetics of methyl esters of labelled isoprostanes according to the radio-TLC (contents of free acid in%)

Basic reagent (1.5 N)	Hydrolysis time, h				
	1	2	3	4	70
LiOH	6	7	-	-	-
K$_2$CO$_3$	16	36	-	-	-
NaOH	74	94	97	99	99.5
KOH	57	88	93	97	97

In summary, we have determined the optimal conditions for introducing of tritium label into rather unstable oxylipins such as leukotrienes and isoprostanes ensuring high yields and specific radioactivities for the labelled compounds. Labelled leukotriene A$_4$ is useful, for example, to study irreversible binding of this reactive intermediate to enzymes involved in its biosynthesis or metabolism, such as 5-lipoxygenase, LTA$_4$ hydrolase and LTC$_4$-synthase (A.Sala, unpublished observations), as well as to study receptor binding. Deuterated isoprostanes provide a very convenient analytical tool for isotope dilution analysis in isoprostane quantification in biological samples while tritiated isoprostanes are important for receptor screening and metabolic studies.

EXPERIMENTAL

Materials. Catalysts, solvents and other reagents were obtained from Fluka (Switzerland); 5% Pd/BaSO$_4$ (Russia) was processed by lead diacetate according to [12]. ^{1}H-NMR spectra were recorded at 360 MHz in CDCl$_3$ previously neutralized over basic aluminum oxide, and ^{13}C-NMR at 90 MHz on a *Bruker* AMX 360.

Preparative separation of reaction mixtures and analysis of purity of compounds were performed using HPLC system Gilson (France) equipped with radioactivity detector and variable wavelength UV-detector. Mass spectra were recorded with Varian Mat-44S (Varian) in the electron impact mode (70 eV). TLC was carried out using silicagel Silufol (Chech Republic) or Kieselgel 60 (Merck, Germany) precoated plates. Optimization of hydrogenation conditions was performed as described previously [13].

Synthesis of 13, 14-dehydro-leukotriene A$_4$ methyl ester (I) (Fig. 1).

A solution of non-3-ynyl triphenyl phosphonium iodide (233 mg, 3 eq, previously dried under vacuum during 15 h with P$_2$O$_5$) in dry THF (2.5 mL) and dry HMPA (500 mL) was stirred under nitrogen at -78 °C. Then *n*-BuLi 1.6 M in hexane (3.6 eq) was added dropwise and the mixture was stirred for 20 min at -78°C (the solution became orange). A solution of methyl 5(S), 6(S)-epoxy-11-oxoundeca-7(E), 9(E)-dienoate (34 mg, 0.089 mmol, 1 equiv) in dry THF (0.5 mL) was added dropwise to the mixture. After 15 min, the solution was concentrated under a reduced pressure at 20°C and immediately purified by flash-chromatography (deactivated silica gel) with heptane/AcOEt/NEt$_3$ 98:2:2, and 40 mg of pure 14,15-dehydro-LTA$_4$ methyl ester (I), 80% yield, were obtained.

UV (ether): l$_{max}$ 267; 277; 288.5 nm. ^{1}H-NMR: 0.87 (t, 3H, H-20, J$_{19-20}$ = 7.0 Hz); 1.22-1.50 (m, 6H, H-17 H-18 H-19); 1.51-1.68 (m, 2H, H-4); 1.70-1.82 (m, 2H, H-3); 2.06-2.17 (m, 2H, H-16); 2.33 (t, 2H, H-2, J$_{2-3}$ = 7.3 Hz); 2.8-2.84 (m, 1H, H-5); 3.00-3.05 (m, 2H, H-13); 3.10 (dd, 1H, H-6, J$_{5-6}$ = 2 Hz, J$_{6-7}$ = 7.9 Hz); 3.64 (s, 3H, H-1'); 5.38 (dd, 1H, H-7, J$_{7-8}$ = 15.4 Hz); 5.47 (td, 1H, H-12, J$_{11-12}$ = 10.3 Hz, J$_{12-13}$ = 7.3 Hz); 6.01 (t, 1H, H-11, J$_{10-11}$ = J$_{11-12}$ = 10.9 Hz); 6.17 (dd, 1H, H-9, J$_{8-9}$ = 10,6 Hz, J$_{9-10}$ = 15.1 Hz); 6.37-6.51 (m, 2H, H-8 H-10).

^{13}C-NMR: 13.9 (C-20); 17.8; 18.7 (C-13 C-16); 21.2 (C-3); 22.2; 28.6; 31.0 (C-17 C-18 C-19); 31.3 (C-4); 33.5 (C-3); 51.5 (C-1'); 58.2 (C-6); 60.4 (C-5); 77.3; 81.0 (C-14 C-15); 127.9; 128.0; 129.0; 130.5; 132.1; 134.3 (C-7 C-8 C-9 C-10 C-11 C-12); 173.6 (C-1).

Synthesis of 15(RS)-5,6-dehydro-8-*epi*-prostaglandin $F_{2\alpha}$ methyl ester (II) (Fig 2).

Tributyltin hydride (47 µl, 170 µmol) was added to a solution of iodinated precursor (51 mg, 72 µmol, in 5 ml of *p*-xylene) under a stream of dry argon. At room temperature, a 1N triethylborane solution (145 µl, 145 µmol) was slowly added under a stream of dry air. The mixture was stirred for 20 min. Triphenylphosphine (38 mg, 145 µmol) was added and the solution was stirred for 2 hours. The mixture was subjected to flash chromatography (0-5% ethyl acetate in cyclohexane) to give 23 mg (55%) of 15(RS)-9,11-O-di(triethylsilyl)-5,6-dehydro-8-epi-PGF$_{2\alpha}$ methyl ester.

UV: (ethanol) l_{max} 204 nm ; ^{1}H NMR: d 0.56 (m, 12H), 0.93 (m, 21H), 1.27 (m, 6H), 1.55 (m, 3H), 1.78 (quint, J = 6.9 Hz, 2H), 2.15 (m, 5H), 2.34 (m, 1H), 2.41 (t, J = 7.5 Hz, 2H), 2.69 (m, 1H), 3.65 (s, 3H), 3.91 (m, 2H), 4.06 (m, 1H), 5.02 (m, 2H) ;

^{13}C NMR: d 4.7, 6.7, 14.0, 18.3, 22.6, 24.2, 25.0, 31.7, 32.9, 37.2, 44.5, 49.0, 51.3, 52.2, 73.0, 74.6, 79.4, 80.1, 129.5, 135.9, 173.6.

A mixture of 15(RS)-9,11-O-di(triethylsilyl)-5,6-dehydro-*8-epi-PGF$_{2\alpha}$* methyl ester (70 mg, 117 µmol) and 1N tetrabutylammonium fluoride solution (176 mmol) was stirred in 2 ml of THF at room temperature for 1 hour. The solution was quenched with 2 ml of brine, and extracted with 3x3 ml of ethyl acetate. The organic layers were combined, washed with 2x2 ml of brine, dried over sodium sulfate and evaporated under reduced pressure. The residue was subjected to flash chromatography (0-4% methanol in methylene chloride) to give 39 mg (90%) of (II).

UV (ethanol): l_{max} 202 nm ; ^{1}H NMR: d 0.87 (m, 3H), 1.27 (m, 6H), 1.50-1.69 (m, 3H), 1.78 (t, J = 7.0 Hz, 2H), 2.05 (m, 2H), 2.19 (t, J = 6.6 Hz, 2H), 2.29 (m, 1H), 2.41 (m, 3H), 2.77 (m, 1H), 3.66 (s, 3H), 4.05 (m, 2H), 4.12 (m, 1H), 5.42 (m, 1H), 5.58 (dd, J = 5.8, 15.3 Hz, 1H) ; ^{13}C NMR: d 13.9, 18.1, 19.1, 22.5, 24.1, 25.1, 31.7, 32.9, 37.3, 42.3, 49.7, 51.6, 53.6, 74.5, 76.1, 76.2, 80.2, 127.7, 136.7, 174.2.

Preparation of catalysts for selective hydrogenation

100 mg of 5% Pd/BaSO$_4$ triturated in a mortar was dispersed in 2 ml of methanol by sonication (1 min). The suspension was treated at room temperature with methanolic solution of lead diacetate (at a Pd/Pb ratio of 0.5 – 2.0) followed by repeated short sonication (20 sec). Sodium borohydride (0,1 – 10 mg) was added to the reaction mixture under vigorous mixing. After the termination of hydrogen

evolution the catalyst was separated by centrifugation, washed out with methanol (3x2 ml), dried at 60-70°C and thoroughly triturated.

Synthesis of [13,14-^{3}H$_2$]-leukotriene A$_4$ methyl ester (Ia)

14,15-Dehydro-LTA$_4$ methyl ester (1 mg), 1 ml of hexane, 12 mg of the catalyst (B) (5% Pd/BaSO$_4$, treated with lead diacetate and sodium borohydride taken in Pd/ Pb ratio of 1:0.7 and ratio of catalyst/NaBH$_4$ of 25:1) and 50 μl of NEt$_3$ were placed in the reaction ampoule and its contents frozen by placing the ampoule in liquid nitrogen. Thr ampoule was evacuated, filled with gaseous tritium to a pressure of 400 hPa, brought up to room temperature and the contents stirred for 30 min. The contents of the ampoule were frozen again and excess of gaseous tritium was removed to a vacuum of 1 mTorr. The catalyst was filtered off and the filtrate was triply evaporated with methanol (3 x 2 ml) to remove labile tritium. Target labelled LTA$_4$ methyl ester (Ia) was isolated by HPLC (column: Separon SGX 5 μm, 3x150 mm; eluent: hexane-ethylacetate-NEt$_3$, 95:5:0.25; elution rate: 0.5 ml/min, UV monitoring at 278 nm). Under these conditions labelled LTA$_4$ methyl ester and its acetylenic precursor showed retention times of 7.45 and 9.37 min, respectively. The radiochemical purity of the labelled LTA$_4$ methyl ester (Ia) improved from 68% to 84% and 97-98% after two stage HPLC purification. The final product was obtained in a yield of 30-35% and specific radioactivity of 40-45 Ci/mmol. The UV spectrum of the labelled (Ia) was in the accordance with its structure and corresponded to the spectrum of the 'cold' LTA$_4$ methyl ester [2].

Synthesis of [5,6-^{3}H$_2$]-8-epi-prostaglandin F$_{2\alpha}$ methyl ester (IIa) and its 15(R)-epimer (IIb). 2 mg of 15(R,S)-5,6-dehydro-8-*epi*-PGF$_{2\alpha}$ (II) in 0.2 ml of hexane-dioxane (1:1) mixture, 50 μl of NEt$_3$ and 6 mg of catalyst (B) were placed into the reaction ampoule and reaction with gaseous tritium was carried out as described above. Labelled 15(S)-8-*epi*-prostaglandin F$_{2\alpha}$ (IIa) and 15(R)-8-*epi*-prostaglandin F$_{2\alpha}$ (IIb) were isolated in an individual state by HPLC (column: Silasorb C$_{18}$ 13 μm, 10x250 mm; eluent: methanol-water, 70:30; elution rate: 2.0 ml/min; UV monitoring at 206 nm, retention times for 15(R)- and 15(S)-epimers were 17.34 and 19.33 min, while acetylenic analogues eluted at 14.02 and 14.97 min, correspondingly). The yield for the sum of 15(R)- and 15(S)-epimers was found to be about 40%, with specific radioactivity of 50-52 Ci/mmol and radiochemical purity of 96–97%. The structures of the labelled isoprostanes were confirmed by mass spectrometry of the corresponding deuterated compounds (that were prepared as described above but replacing gaseous tritium with deuterium).

Synthesis of [5,6-^{3}H$_2$]-8-*epi*-prostaglandin F$_{2\alpha}$ and its 15(R)-epimer.

One hundred microliters of solution of labelled (**IIa**) or its 15(R)-epimer (**IIb**) in a mixture of methanol-water, 3:7 (2 mCi/ml) was treated with 20 μl of 1.5 N aqueous NaOH (or other alkali hydroxide), and the mixture was kept at 20°C for 4 h (or in accordance with Table 3). Reaction progress was followed by TLC using chloroform-methanol-conc. ammonia, 80:20:1, R$_f$ [8-epi-PGF$_{2\alpha}$] 0.1 – 0.15, R$_f$ [8-epi-PGF$_{2\alpha}$ methyl ester] 0.75 – 0.8. The radiochemical purity of 95 - 97% (was determined by TLC using chloroform-methanol-acetic acid, 90:9:1, R$_f$ [8-epi-PGF$_{2\alpha}$ (both epimers)] 0.5) and specific radioactivity of 50 Ci/mmol were found .

Acknowledgements

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