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A CONVENIENT SYNTHESIS OF [3-^{3}H]SQUALENE AND [3-^{3}H]-2,3-OXIDOSQUALENE

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SUMMARY

[3-^{3}H]Squalene and [3-^{3}H]-2,3-oxidosqualene, key compounds for studying the biosynthesis of sterols, were synthesized. The main step was a modified Wittig reaction of [1-^{3}H]trisnorsqualene aldehyde with a phosphorus ylide to give [3-^{3}H]squalene or with a sulfur ylide to give [3-^{3}H]-2,3-oxidosqualene. These compounds were obtained in high radiochemical yield and high purity under simple conditions.

Key words: squalene epoxidase, oxidosqualene cyclase, tritium labelling, cholesterol biosynthesis, enzyme assay.

INTRODUCTION

Squalene epoxidase (EC 1.14.99.7) and oxidosqualene-lanosterol cyclase (EC 5.4.99.7) are two enzymes involved in the epoxidation of squalene to 2,3-oxidosqualene, and subsequent cyclization to the tetracyclic triterpene lanosterol, the first cyclized sterol precursor in animals and fungi. In studying the mechanism of enzymatic cyclization and the activity of related inhibitors, [^{3}H] or [^{14}C] labelled substrates are employed (1,2 and references therein).

To our knowledge, no convenient syntheses of [3-^{3}H]squalene have been reported until now. So we decided to label chemically squalene with tritium at a specific position (C-3) via an intermediate trisnorsqualene aldehyde (1).

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Most syntheses of [^{3}H], [^{13}C] and [^{14}C]squalene are based on coupling labelled precursors (3,4). The method of Wolff and Pichat starts from $Ba^{14}CO_3$ and gives through a Wittig reaction [11,14-$^{14}C_2$]squalene (5). The Biellmann and Ducep method, based on reaction of farnesyl phenyl sulphide carbanion with tritium labelled water, gives C-11 and C-12 [^{3}H]squalene (6). [4-^{3}H]Squalene was prepared by Nadeau and Hanzlik (7) by reacting trisnorsqualene aldehyde (1), with a [$^{3}H^{+}$] solution in tritiated water (1 Ci/ml). Recently, total synthesis of [10,15-$^{13}C_2$]squalene starting from [3-^{13}C] ethyl farnesoate has been accomplished in various steps (8).

Many of the described methods for the synthesis of radiolabelled squalene can also be applied to the corresponding 2,3-epoxide, while others give directly the epoxide. [1-^{14}C]-2,3-oxidosqualene was obtained by Pichat in high specific radioactivity by reacting trisnorsqualene aldehyde (1) with the ylide of [^{14}C]diphenylisopropylsulphonium tetrafluoroborate (9). [10,15-$^{13}C_2$]-2,3-oxidosqualene has been obtained with a total synthesis in 1.6% overall yield (8), while racemic and [24,30-^{14}C]-(3S)-2,3-oxidosqualene have been obtained in an elegant way (10). A synthesis of [11,14-$^{3}H_2$]-2,3-oxidosqualene started from the corresponding labelled squalene (11).

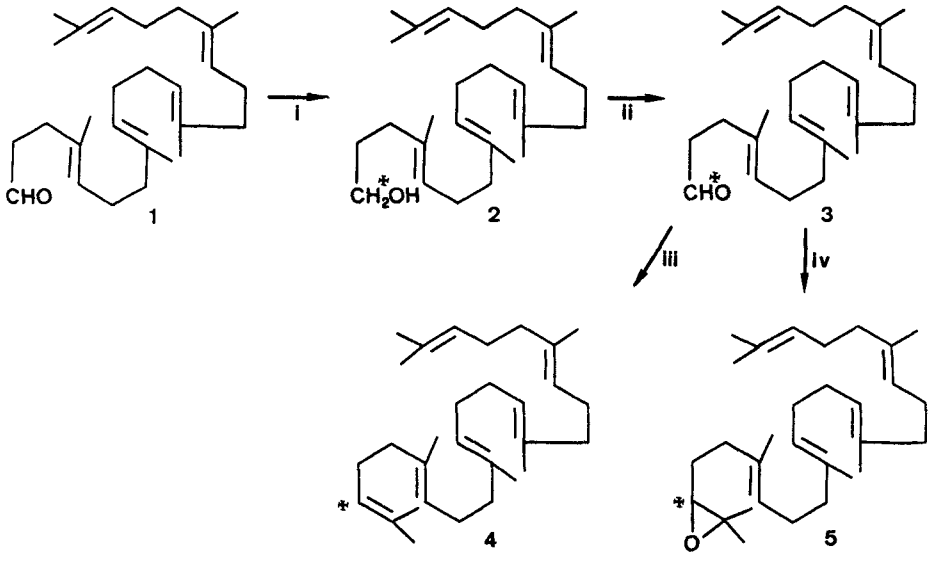
The reported syntheses of labelled squalene and the corresponding 2,3-epoxide were based on complex synthetic procedures characterized by low overall yields.

There are several advantages in using tritium over [^{14}C]. These include the usually higher molar radioactivity of tritium labelled compounds than their [^{14}C] analogs and the easy handling of this isotope with respect to [^{14}C]. For studies on squalene epoxidase, commercial [^{3}H] or [^{14}C] squalene are usually used. These compounds, available in small amounts, labelled in six positions can be biologically prepared by incubations with [^{3}H] or [^{14}C]mevalonic acid (12,13) or with other labelled precursors.

Here we report a simple method for synthesizing both [3-^{3}H]squalene and [3-^{3}H]-2,3-oxidosqualene, based on a Wittig reaction between [1-^{3}H]trisnorsqualene aldehyde and the suitable ylide. They are obtained in high yield and high purity under simple conditions.

RESULTS AND DISCUSSION

Trisnorsqualene aldehyde (1) was obtained according to a method developed by us, based on reaction of squalene with N-bromosuccinimide, closure to 2,3-epoxide with K_2CO_3 and subsequent cleavage with $HIO_4.2H_2O$ in diethyl ether (14,15). Trisnorsqualene aldehyde (1) was reduced with NaB^3H_4 in methanol to [1-^{3}H]trisnorsqualene alcohol (2) and directly retransformed to [1-^{3}H]trisnorsqualene aldehyde (3) with pyridinium chlorochromate (PCC) in CH_2Cl_2 (Scheme I). A more commonly used method to oxidize sterols or precursors is the use of CrO_3-pyridine complex (16). The use of PCC makes the reaction simple and easy to work up. Wittig reaction under reflux with the instant ylide [$(CH_3)_2CHP^+Ph_3Br^-$ + $NaNH_2$ + BuLi in THF] afforded [3-^{3}H]squalene (4) in 84% yield.



Scheme I. * indicates position of ^{3}H-label

(i) NaB^3H_4, CH_3OH; (ii) PCC, CH_2Cl_2; (iii) [$(CH_3)_2CHP^+Ph_3Br^-$, $NaNH_2$, BuLi, THF]; (iv) [$(CH_3)_2CHS^+Ph_2BF_4^-$, PhLi, THF].

According to the literature, the ylide of isopropyltriphenylphosphonium bromide or iodide is generated at 0 °C or room temperature and then reacted in the case of aliphatic aldehydes usually at 0 °C or room temperature for a 10 min - 24 h period. In our case, following these conditions

(7,17,18), we obtained low amounts of [3-^{3}H]squalene (4). The use of diethyl ether instead of THF at room temperature gave similar results. Reflux of the ethereal solution of the instant ylide [$(CH_3)_2CHP^+Ph_3Br^-$ + $NaNH_2$ + BuLi] for 15 min - 2 h periods gave to a reasonable yield (25-35%) of [3-^{3}H]squalene (4), while reflux of the THF solution of the instant ylide for 15 min afforded [3-^{3}H]squalene (4) in 84% radiochemical yield.

It is interesting to observe that at room temperature the chemical and radiochemical yields of squalene were different, as the chemical yields were in the 30-40% range, while the radiochemical yields, although not very reproducible, were in the 6-15% range. This might be due to an isotopic effect during the Wittig reaction.

The synthesis of [1-^{3}H]trisnorsqualene aldehyde (3) here described has now also been applied to the synthesis of [3-^{3}H]-2,3-oxidosqualene (5). The sulfur ylide of diphenylisopropylsulphonium tetrafluoroborate was generated with phenyllithium in THF and reacted with [1-^{3}H]trisnorsqualene aldehyde (3) to give [3-^{3}H]-2,3-oxidosqualene (5).

The main chemical advantages of the described syntheses are the following:

1) they start from trisnorsqualene aldehyde (1), easily obtainable in the desired amounts (14).
2) they avoid the use of tritiated water, which is tedious to use.
3) [3-^{3}H]squalene (4) and [3-^{3}H]-2,3-oxidosqualene (5) can be obtained with high specific radioactivity, depending on the specific radioactivity of the NaB^3H_4 used, and in high radiochemical yield.
4) [3-^{3}H]squalene (4) is obtained without TLC purification, as two rapid short column chromatography purifications are used. In addition, the use of non-volatile labelled compounds makes this method particularly safe.

EXPERIMENTAL

^{1}H NMR spectra were recorded on a Jeol EX-400, with $SiMe_4$ as internal standard. Mass spectra were obtained on a VG Analytical 7070 EQ-HF or a VG ZAB 2F spectrometer. Light petroleum refers to the fraction of bp 40-60 °C. THF was distilled under sodium benzophenone ketyl.

For determination of the radioactive substances formed, samples of the crude and purified products were eluted on F_{254} silica gel precoated sheets. The radioactive bands were visualized with a Packard radiochromatogram reader. The sheets were then exposed to iodine vapour to show the correspondence between the tritium labelled compound and a cold sample. For determination of specific and total radioactivity, three samples of each pure product, diluted in benzene, were counted for radioactivity in a Beckman LS 5000 liquid scintillator. Isotope counting was carried out as already described and the mean value was determined (19).

Pure [3-^{3}H]squalene and [3-^{3}H]-2,3-oxidosqualene can be stored for a long time diluted and frozen in dry benzene.

[1-^{3}H]Trisnorsqualene alcohol: [1-^{3}H]-(4E,8E,12E,16E)-4,8,13,17,21-pentamethyl-4,8,12,16,20-docosapentaen-1-ol (2)

Pure trisnorsqualene aldehyde (1), obtained as described in ref. (14) (10 mg, 0.026 mmol) was dissolved in methanol (500 µl) and added to the phial containing NaB^3H_4 (total activity 100 mCi, specific activity 10 Ci/mmol, total amount 0.01 mmol). After 1 h, $NaBH_4$ (excess, 3.9 mg, 0.104 mmol) was added to complete the reaction. After an additional 1 h, the methanol was evaporated under nitrogen and the reaction mixture was dissolved in dichloromethane, transferred to a single necked flask, filtered to eliminate suspended $NaBH_4$ and evaporated to dryness *in vacuo* to give 7.54 mg (0.0195 mmol) of [1-^{3}H]trisnorsqualene alcohol (2).

The radiochemical and chemical purity of the crude alcohol was determined by radiochromatogram with cyclohexane / ethyl acetate, 85:15 and then revealed with iodine vapour. The TLCs, with various eluants, of [1-^{3}H]trisnorsqualene alcohol (2) were identical to those of an authentic sample.

^{1}H-NMR and IR data of trisnorsqualene alcohol from reaction with radio-inert reagents:

^{1}H-NMR ($CDCl_3$) δ: 1.58-1.72 (m, 20 H, allylic CH_3 and $C\underline{H}_2CH_2OH$), 1.96-2.10 (m, 18 H, allylic CH_2), 3.65 (t, 2 H, $CH_2\underline{O}H$), 5.01-5.17 (m, 5H, vinylic CH).

IR (neat): 3330, 2925, 1665 cm^{-1}.

Total activity: 75 mCi; specific activity: 3.8 Ci/mmol; chemical yield 75% (of the crude product).

The crude product, chemically and radiochemically pure was used directly in the next step.

[1-^{3}H]Trisnorsqualene aldehyde: [1-^{3}H]-(4E,8E,12E,16E)-4,8,13,17,21-pentamethyl-4,8,12,16,20-docosapentaenal (3)

Pyridinium chlorochromate (PCC) (x 5, 0.097 mmol, 20.9 mg) was dissolved in a single necked flask in dichloromethane (300 μl). The crude [1-^{3}H]trisnorsqualene alcohol (2) (total activity 75 mCi, 7.54 mg, 0.0195 mmol) was dissolved in dichloromethane (200 μl) and added to the solution. Further dichloromethane (200 μl) was added twice to transfer all the alcohol. The reaction mixture was allowed to react 90 min at room temperature under nitrogen, with stirring. During this time the color progressively changed to brown with a dark brown precipitate. The reaction mixture was evaporated and diethyl ether was added (5 ml). The ethereal solution was transferred in another single necked flask and additional ether (5 ml x 3) was added to the reaction mixture. The brown solid was discarded. The ethereal extracts were evaporated to dryness and added of light petroleum (5 ml). The solution of the crude aldehyde was purified by column chromatography (10 cm height, 2 cm diameter) on silica gel (70-230 mesh) with light petroleum / diethyl ether, 99.5:0.5 to remove impurities, then light petroleum / diethyl ether 99:1 (10 fractions of 30 ml) to give pure [1-^{3}H]trisnorsqualene aldehyde (3) (6.89 mg, 0.0179 mmol).

The radiochemical and chemical purity was determined by radiochromatogram with cyclohexane / ethyl acetate, 85:15 and then revealed with iodine vapour. The TLCs, with various eluants, of [1-^{3}H]trisnorsqualene aldehyde (3) were identical to those of an authentic sample.

^{1}H-NMR and IR data of trisnorsqualene aldehyde from reaction with radio-inert reagents:
^{1}H-NMR ($CDCl_3$) δ: 1.56-1.72 (m, 18 H, allylic CH_3), 1.94-2.09 (m, 18 H, allylic CH_2), 2.33-2.40 (m, 2 H, $C\underline{H}_2CHO$), 5.01-5.21 (m, 5H, vinylic CH), 9.71 (m, 1 H, CHO).
IR (neat): 2980, 2910, 2850, 1730 (CO), 1450 and 1385 cm^{-1}.

These data were identical to those of trisnorsqualene aldehyde from reaction of 2,3-oxidosqualene with $HIO_4.2H_2O$ in diethyl ether.

Total activity: 34 mCi; specific activity: 1.9 Ci/mmol; chemical yield 92% [from [1-^{3}H]trisnorsqualene alcohol (2)].

[3-^{3}H]Squalene: [3-^{3}H]-(6E,10E,14E,18E)-2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene (4)

Following several reactions with unlabelled reagents, isopropyltriphenylphosphonium bromide plus sodium amide (great excess, 70 mg, 0.16 mmol of phosphonium salt; 1 g = 2.3 mmol of phosphonium bromide, Fluka) was suspended in dry THF (5 ml) and stirred in an oil bath at reflux under a stream of dry nitrogen. Then, BuLi (about 1.6 M solution in hexane, x 1.0 with respect to phosphonium salt, 0.16 mmol, 100 μl) was slowly added. During this addition the color progressively turned to red. Pure [1-^{3}H]trisnorsqualene aldehyde (3) (total activity 34 mCi, 6.89 mg, 0.0179 mmol) dissolved in diethyl ether (100 μl) was then slowly added. After 15 min stirring at reflux, in an oil bath at 80 °C, the reaction mixture was poured into a biphasic system (light petroleum / brine, 1:1, 30 ml), extracted with light petroleum (20 ml x 2) and the organic layers were washed with brine (20 ml x 2), dried and evaporated in vacuo. The crude product was purified by column chromatography (12 cm height, 2 cm diameter) on silica gel (70-230 mesh) with n-heptane as eluant, to give pure [3-^{3}H]squalene (4) (6.16 mg, 0.0150 mmol).

The radiochemical and chemical purity was determined by radiochromatogram with light petroleum and then revealed with iodine vapour. The TLCs, with various eluants, of [3-^{3}H]squalene (4) were identical to those of an authentic sample.

^{1}H-NMR, EIMS and HRMS data of squalene from reaction with radio-inert reagents:

^{1}H-NMR ($CDCl_3$) δ: 1.59-1.69 (m, 24 H, allylic CH_3), 1.97-2.11 (m, 20 H, allylic CH_2), 5.03-5.19 (m, 6H, vinylic CH).

EIMS: m/z 410 (7), 367 (4), 341 (10), 328 (2), 299 (3), 273 (5), 259 (2), 231 (4), 218 (3), 203 (6), 191 (7), 149 (17), 137 (35), 81 (65), 69 (100).

HRMS: 410.3917 (calc. for $C_{30}H_{50}$, 410.3912).

These data were identical to those of an authentic sample.

Total activity: 28 mCi; specific activity: 1.9 Ci/mmol; chemical yield 84%.

[3-^{3}H]-2,3-Oxidosqualene: [22-^{3}H]-(6E,10E,14E,18E)-22,23-epoxy-2,6,10,15,19,23-hexamethyl-2,6,10,14,18-tetracosapentaene (5)

Diphenylisopropylsulphonium tetrafluoroborate (7) (x 3, 17.1 mg, 0.054 mmol) was added in a two-necked flask of dry THF

(1 ml), kept under dry nitrogen, stirred and cooled at -70 °C. Then freshly prepared phenyllithium (about 2 M solution in benzene / diethyl ether, 70:30; excess, 100 μl) was slowly added, while the colorless solution turned to yellow-orange. After 5 min, pure [1-^{3}H]trisnorsqualene aldehyde (3) (total activity 34 mCi, 6.89 mg, 0.0179 mmol) in THF (200 μl) was added. The reaction mixture was kept 1 h at -70 °C, then allowed to reach room temperature. The reaction mixture was then diluted with n-hexane (20 ml) and poured into water (20 ml). It was then extracted with n-hexane (20 ml x 2), washed with brine (10 ml x 2), dried and evaporated in vacuo. The crude oil was diluted with dichloromethane (100 μl) and purified on silica TLC (0.25 mm) with benzene / dichloromethane, 50:50 to give pure [3-^{3}H]-2,3-oxidosqualene (5) (5.89 mg, 0.0138 mmol).

The radiochemical and chemical purity were determined by radiochromatogram with benzene / dichloromethane, 50:50 and then revealed with iodine vapour. The TLCs, with various eluants, of [3-^{3}H]-2,3-oxidosqualene (5) were identical to those of an authentic sample.

^{1}H-NMR, EIMS and HRMS data of 2,3-oxidosqualene from reaction with radio-inert reagents:

^{1}H-NMR ($CDCl_3$) δ: 1.24 and 1.28 (two peaks, 6 H, epoxidic CH_3), 1.58-1.66 (m, 20 H, allylic CH_3 and CH_2-epoxide), 1.98-2.06 (m, 18 H, allylic CH_2), 2.69 (t, 1 H, epoxidic CH), 5.06-5.17 (m, 5H, vinylic CH).

EIMS: m/z 426 (5), 409 (3), 383 (2), 357 (5), 339 (2), 315 (2), 299 (2), 289 (3), 271 (5), 203 (12), 191 (11), 153 (21), 135 (40), 69 (100).

HRMS: 426.3858 (calc. for $C_{30}H_{50}O$, 426.3861).

These data were identical to those of 2,3-oxidosqualene from reaction of squalene monobromohydrin with K_2CO_3 in methanol.

Total activity: 26 mCi; specific activity: 1.9 Ci/mmol; chemical yield 77%.

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