# Two new convenient syntheses of 14C-squalene from turbinaric acid



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## Abstract

Carbon-14 labelled (6E,10E,14E,18E)-2,6,10,15,19,23-hexamethyltetracosa-2,6,10,14,18,22-hexaene, also known as squalene, was synthesized as a tool for pharmacokinetic studies. Two simple and efficient labelling approaches were developed to give [2-$^{14}$C]-Squalene and [3-$^{14}$C]-Squalene from a halogenated precursor derived from turbinaric acid. They were obtained in 13.5% radiochemical yield in 6 steps and in 38% radiochemical yield in 3 steps respectively from carbon-14 labelled potassium cyanide with a radiochemical purity higher than 98% in both cases.

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## Introduction

Squalene **1** is a triterpenoid aliphatic hydrocarbon, naturally produced by all plants and animals including humans (figure 1), for which it is an essential biosynthetic precursor of cholesterol. It is also used for cosmetic purposes, as a dietary supplement and as an adjuvant in vaccines. In addition, **1** shows protective and preventive effects in cancer and in the treatment of other diseases[1]. For potential pharmaceutical applications, carbon-14 labelled squalene would be a useful radioactive probe for pharmacokinetic studies.

To our knowledge, only a very few syntheses of isotopically labelled squalene and its derivatives have been published: the synthesis of [11, 12-$^{14}C_2$]-squalene and of [1-$^{14}$C]-2,3-oxidosqualene was reported by Pichard et al.[2,3] in 1958 and in 1973, the preparation of [24,30-$^{14}$C]-2,3-oxidosqualene was described by Prestwich et al.[4] in 1991, a convenient synthesis of [3-$^{3}$H]-squalene was published by L.Cattel et al.[5] in 1994 and the preparation of ω-di-(trideuteromethyl)-squalene was reported by D. Desmaële et al.[6] in 2016. We now describe simple syntheses of [$^{14}$C]-squalene **2** and **3**, labelled at two specific positions (C-2) and (C-3) respectively, starting from turbinaric acid[7] **4**, readily available in high purity within our company. The latter **4** was obtained in 4 steps from commercially available squalene **1** according to the synthesis described by J. Mann et al.[8]. The synthesis of [2-$^{14}$C]-squalene **2** was performed using a novel approach whereas the synthetic route leading to [3-$^{14}$C]-squalene **3** was developed using a Wittig reaction at the last step, as described in the references above.

## Results and discussion

For the synthesis of [2-$^{14}$C]-Squalene **2** (scheme 1), turbinaric acid **4** was reduced to the alcohol **5** with lithium aluminium hydride in 90% yield. Conventional iodination of **5** with iodine in the presence of triphenylphosphine and imidazole resulted in the corresponding halogeno intermediate **6** in good yield. The latter was submitted to cyanation with carbon-14 potassium cyanide in DMSO to give the [$^{14}$C]-nitrile **7** in 94% radiochemical yield. **7** was then treated with an excess of methyl lithium to give the ketone **8** in only 25% yield although during unlabelled development work, this step proceeded in an excellent 90% yield. **8** was transformed to tertiary alcohol **9** with methylmagnesium iodide in 90% radiochemical yield. At this point, an alternative synthetic route was developed to increase the overall yield of **9** from the [$^{14}$C]-cyano compound **7**. The first step consisted of hydrolyzing **7** under strong alkaline conditions to obtain the corresponding acid **10** in 94% yield. **10** was then activated with 1,1'-carbonyldiimidazole and reacted with methanol to give methyl ester **11** in 92% yield. **11** was finally treated with an excess of methylmagnesium iodide to give **9** in 75% radiochemical yield over 3 steps. In the last step, **9** was dehydrated with Burgess reagent to give both expected elimination products: [2-$^{14}$C]-squalene **2** and terminal ethylenic isomer **12**. Proton NMR analysis showed that the **2**/**12** ratio was disappointingly poor (55/45). So, different methods to remove the unwanted isomer **12** were tested such as its degradation by 9-BBN[9] or purification on classical semi-preparative or specific Silica-gel-$AgNO_3$[10] columns. Only the latter allowed us to efficiently recover over half of the radioactivity of [2-$^{14}$C]-squalene **2** with a radiochemical purity higher than 98% after multiple chromatographic runs. NMR analysis revealed a contamination of [2-$^{14}$C]-squalene **2** with significant amounts of butylated hydroxytoluene (BHT) coming from solvents used during the synthesis, however this was easily removed through a final purification on a RP18 column to

give the [2-$^{14}$C]-squalene **2** in 13.5% overall radiochemical yield from carbon-14 potassium cyanide.

A second approach, quicker and more convenient, was developed to obtain [3-$^{14}$C]-squalene **3** from turbinaric acid **4** as depicted in scheme 2. **4** was bromodecarboxylated under Barton's conditions[11] to give the bromo derivative **13** in 34% yield. **13** was converted into the radioactive nitrile **14** with carbon-14 potassium cyanide in dimethylsulfoxide in 58% yield then reduced with diisobutylaluminium hydride solution to aldehyde **15.** Finally, **15** was reacted with the ylide of isopropyltriphenylphosphonium bromide using the conditions described by L. Cattel et al.[5] to give [3-$^{14}$C]-squalene **3** in 65% radiochemical yield in 2 steps and a radiochemical purity higher than 98%.

## Experimental details

**General:**
Carbon-14 labelled potassium cyanide (specific activity 2109MBq/mmol) was purchased from Tjaden Bioscience, (Burlington, IA, USA). Pure turbinaric acid was obtained from the Chemical Development department of Sanofi R&D. All reagents and solvents were purchased from commercial suppliers and used without further purification.
All experimental procedures were optimized using unlabeled materials. Air and moisture sensitive reactions were conducted under an inert atmosphere of nitrogen and were magnetically stirred. Reactions were monitored by HPLC and thin layer chromatography (TLC) which was performed on 60 $F_{254}$ silica gel plates. Radiolabeled products were compared with authentic materials. Merck silica radio-TLC plates were analyzed and quantified by electronic autoradiography using a Packard Instant imager. Plates were exposed to iodine vapor to show the correspondence between radiolabeled and unlabeled products. Quantification of radioactivity was determined using Ultima Gold as liquid scintillation cocktail on a Perkin Elmer TRI-CARB ® 2900TR liquid scintillation counter.
Proton NMR spectra were recorded on a Bruker Avance 600 spectrometer in the stated deuterated solvent.

HPLC analyses were performed on a Shimadzu Prominence UFLC system equipped with a diode array UV detector and a Packard Radiomatic 500TR series flow scintillation analyser. The following chromatographic conditions were used: Akzo Nobel Kromasil C18 (5µm particles – 250mm length x4.6mm internal diameter) column, isocratic elution at room temperature, mobile phase: acetonitrile with 5% water, flow rate: 1ml/min, UV wavelength: 220nm.

**Preparation of the Silica – $AgNO_3$ [12]:**
900g of silica gel (Art Merck 9385) were dried in an oven at 200°C for 8 hours. 150g of this silica were poured in amber 250ml round bottom flasks and 30g of $AgNO_3$ in 40ml of water were added slowly with vigorous stirring between each addition. Place the flask in the rotary evaporator and rotate for 1 hour under atmospheric pressure and room temperature. The silica–$AgNO_3$ gel thus obtained was stocked in amber flasks under argon in darkness.

## EXPERIMENTAL PROCEDURE

## Radiosynthesis of [2-$^{14}$C]-squalene (2)

### (4E,8E,12E,16E)-4,8,13,17,21-pentamethyldocosa-4,8,12,16,20-pentaenol (5)

A stirred solution of turbinaric acid **4** (1.60g, 4mmol) in diethyl ether (250ml) was treated slowly with $LiAlH_4$ (0.25g, 6.6mmol) at 0°C and the mixture was stirred for 1 hour at room temperature. After cooling to 0°C, a saturated aqueous solution of $Na_2SO_4$ was added to destroy excess hydride. The organic solution was poured into a separating funnel and extracted twice with diethyl ether (150ml). The combined organic solution was dried over $MgSO_4$, filtered and concentrated under vacuum. The oil was dissolved in a mixture of n-pentane/diethyl ether (1:1, v/v) (4ml) and purified by chromatography on a silica gel column eluted with a mixture of n-pentane/diethyl ether (6:4, v/v) to afford **5** (1.39g, 90%) as colorless oil. TLC ($SiO_2$, diethyl ether/n-pentane (6:4, v/v), $R_f$ = 0.35).
$^1$H-NMR (600 MHz, $CDCl_3$) δ 5.08 to 5.20 (m, 5H), 3.64 (t, J=6.42 Hz, 2H), 2.04 to 2.13 (m, 8H), 1.96 to 2.04 (m, 10 H), 1.69 (s, 3H), 1.68 (m including J=6.40 Hz, 2H), 1.63 (s, 3H), 1.61 (m, 12H)

### (6E,10E,14E,18E)-22-iodo-2,6,10,15,19-pentamethyldocosa-2,6,10,14,18-pentaene (6)

A stirred solution of **5** (1.35g, 3.5mmol) in dichloromethane (200ml) was treated with imidazole (0.255g, 3.75mmol), triphenylphosphine (0.984g, 3.75mmol) and iodine (0.965g, 3.8mmol). At the end of the addition, the reaction was allowed to stir at room temperature for 1 hour. Water (100ml) was added and the organic layer separated, washed again with water (100ml), dried over $MgSO_4$, filtered and concentrated under vacuum. The residue was taken up in a minimum of pentane and purified by chromatography using a silica gel column eluted with n-pentane to afford **6** (1.56g, 90%). TLC ($SiO_2$, diethyl ether/n-pentane (15:85, v/v), $R_f$ = 0.85).
$^1$H-NMR (600 MHz, $CDCl_3$) δ5.09 to 5.21 (m, 5H), 3.15 (t, J=7.02 Hz, 2H), 2.05 to 2.13 (m, 8H), 1.97 to 2.05 (m, 10H), 1.92 (quint, J=7.02 Hz, 2H) 1.70 (s, 3H), 1.62 (m, 3H), 1.61 (s, 9H), 1.60 (s, 3H)

### [1-$^{14}$C]-(5E,9E,13E,17E)-5,9,14,18,22-pentamethyltricosa-5,9,13,17,21-pentaenenitrile (7)

A stirred suspension of [$^{14}$C]-Potassium cyanide (3.7GBq, 1.75mmol) in dimethylsulfoxide (15ml) was treated with a solution of **6** (1g, 2mmol) in dimethylsulfoxide (5ml) and the mixture stirred at room temperature overnight. Water (100ml) was added and the product was extracted twice with diethyl ether (150ml). The combined organic solution was dried over $MgSO_4$, filtered and concentrated under vacuum. The residue was taken up in a minimum of diethyl ether/n-pentane (1:1, v/v) and purified by chromatography using a silica gel column eluted with diethyl ether/n-pentane (15:85, v/v) to afford **7** (3.478GBq, 94% radiochemical yield) as colorless oil. TLC ($SiO_2$, diethyl ether/n-pentane (15:85, v/v), $R_f$ = 0.80).

### [2-$^{14}$C]-(6E,10E,14E,18E)-6,10,15,19,23-pentamethyltetracosa-6,10,14,18,22-pentaen-2-one (8)

A stirred solution of **7** (3.14 GBq, 1.5 mmol) in anhydrous diethyl ether (50ml) was treated slowly with a 1.6M solution of methyl lithium in diethyl ether (1.5ml, 2.4mmol) at 0°C. The mixture was stirred at room temperature for 2h. The flask was cooled in an ice bath and treated

with a saturated aqueous solution of $NH_4Cl$ (20ml). The product was extracted twice with diethyl ether (20ml). The combined organic layers were dried over $MgSO_4$, filtered and concentrated. The residue was taken up in a minimum of pentane and purified by chromatography using a silica gel column eluted with diethyl ether/n-pentane (25:75, v/v) to afford **8** (787.5 MBq, 25% radiochemical yield) as a colorless oil. TLC ($SiO_2$, diethyl ether/n-pentane (25:75, v/v), $R_f$ = 0.75)

**[2-$^{14}$C]-(6E,10E,14E,18E)-2,6,10,15,19,23-Hexamethyltetracosa-6,10,14,18,22-pentaen-2-ol (9)**

**From Ketone compound (8)**

A stirred solution of **8** (787.5 MBq, 0.37mmol) in anhydrous diethyl ether (20ml) was treated slowly with a 3M methyl magnesium iodide solution in diethyl ether (0.3 ml, 0.9mmol) at 0°C. The mixture was stirred at room temperature for 1h. The flask was cooled in an ice bath and treated with a saturated aqueous solution of $NH_4Cl$ (10ml). The product was extracted twice with diethyl ether (15ml). The combined organic phases were dried over $MgSO_4$, filtered and concentrated. The residue was dissolved in a minimum of n-pentane and purified by chromatography using a silica gel column eluted with diethyl ether/n-pentane (from 1:1 to 8:2) to afford **9** (708.8 MBq, 90% radiochemical yield) as a colorless oil. TLC ($SiO_2$, ethyl acetate/n-pentane (7:3, v/v), $R_f$ = 0.30)

**From ester compound (11)**

A stirred solution of **11** (3.071GBq, 1.4mmol) in anhydrous diethyl ether (80ml) was treated slowly with a 3M methyl magnesium iodide solution in diethyl ether (1.5ml, 4.5mmol) at 0°C. The mixture was stirred at room temperature overnight. The flask was cooled in an ice bath and treated with a satured aqueous solution of $NH_4Cl$ (50ml). The product was extracted twice with diethyl ether (100ml). The combined organic phases were dried over $MgSO_4$, filtered and concentrated. The residue was dissolved in n-pentane and purified by chromatography using a silica gel column eluted with diethyl ether/n-pentane (from 1:1 to 8:2, v/v) to afford **9** (2.664GBq, 86.7% radiochemical yield) as a colorless oil. TLC ($SiO_2$, ethyl acetate/n-pentane (7:3, v/v), $R_f$ = 0.30)

**[1-$^{14}$C]-(5E,9E,13E,17E)-5,9,14,18,22-pentamethyltricosa-5,9,13,17,21-pentaenoic acid (10)**

A stirred solution of **7** (3.478 GBq, 1.65mmol) in ethanol (35ml) was treated slowly with a solution of potassium hydroxide (14g, 0.24mol) in water (35ml). The mixture was heated under reflux for 6h. The reaction was monitored by HPLC ($R_t$ acid: 9.7 min - $R_t$ amide: 10.6 min according to the above conditions).The reaction was allowed to cool to room temperature overnight and concentrated under vacuum. The residue was dissolved in water (150ml) and acidified by the addition of citric acid to pH= 5. The mixture was extracted twice with ethyl acetate (150ml). The combined organic solution was dried over $MgSO_4$, filtered and concentrated in vacuum.

The residue was dissolved in ethyl acetate (4ml) and purified by chromatography using a silica gel column eluted with a mixture of ethyl acetate/n-pentane (from 5:5 to 8:2, v/v) to afford **10** as a colorless oil. Radio-TLC analysis showed 94% radiochemical yield. TLC ($SiO_2$, ethyl acetate/n-pentane (6:4, v/v), $R_f$ = 0.35). HPLC ($R_t$ acid: 9.7 min)

**[1-$^{14}$C]-(5E,9E,13E,17E)-5,9,14,18,22-Pentamethyltricosa-5,9,13,17,21-pentaenoic acid methyl ester (11)**

N,N-Carbonyl diimidazole (0.324g, 2mmol) was added to a solution of **10** (3.304GBq,1.56mmol) in tetrahydrofuran (50ml). The mixture was refluxed under stirring for 1h. The reaction was monitored by HPLC ($R_t$ amide: 12.6min according to above conditions). When no more acid was detected by HPLC, methanol (20ml) was added and the reflux maintained for 4h. The esterification was monitored by HPLC ($R_t$ amide: 12.6min - $R_t$ ester: 14.6min according to above conditions). The reaction was allowed to cool to room temperature and concentrated in vacuum. Diethyl ether (200ml) was added and washed twice with water (150ml). The organic solution was dried over $MgSO_4$, filtered and concentrated in vacuum. The residue was taken up in a minimum of pentane and purified by chromatography using a silica gel column eluted with n-pentane to afford **11** (3.071GBq, 92% radiochemical yield) as oil. TLC ($SiO_2$, diethyl ether/n-pentane (85:15, v/v), $R_f$ = 0.75).

**[2-$^{14}$C]-(6E,10E,14E,18E)-2,6,10,15,19,23-Hexamethyltetracosa-6,10,14,18,22-hexaene (2)**

- A stirred solution of **9** (2.664GBq, 1.26mmol) in diethyl ether (25ml) was treated with Burgess reagent (0.476g, 2mmol, CAS Number : 29684-56-8) at -20°C for 15mn. The reaction was monitored by HPLC ($R_t$ alcohol: 14.9 min - $R_t$ squalene + isomer: 30.4 min according to above conditions). After 14 hours, the mixture was diluted with diethyl ether (50ml) and a brine solution (10ml) was added. The product was extracted twice with diethyl ether (150ml). The combined organic solution was dried over $MgSO_4$, filtered and concentrated. The residue was dissolved in pentane (5ml) and purified by chromatography using a silica gel column eluted with diethyl ether/n-pentane (1:9, v/v) to afford a mixture of two hexaenes in a [2-$^{14}$C]-squalene **2**/(6e,10e,14e,18e)-1,6,10,15,19,23-Hexamethyltetracosa-6,10,14,18,22-[2-$^{14}$C]-hexaene **12** ratio of 55%/45% as oil (2.62GBq, 98% yield ). The ratio was determined by proton NMR spectrum integration of ethylenic signals (characteristic isolated doublet at 4.63 ppm belonging to the unwanted isomer **12**).

**Purification of [2-$^{14}$C]-squalene (2)**

The oily residue from the above experiment (1.78GBq) was purified by chromatography, successively through three columns of silica gel-$AgNO_3$ (200g) eluted with n-pentane/ethyl acetate (1:1, v/v) to give 555 MBq of crude **2** with a stereoisomeric purity of 98,7% measured by proton NMR. A final purification on a RP18 column, using a mixture of acetonitrile - tetrahydrofuran (freshly distilled) was necessary to eliminate traces of BHT to afford 495MBq of [2-$^{14}$C]-squalene **2** as a colorless oil (27% yield), 99.9% radiochemical purity determined by HPLC according to above conditions). $^1$H-NMR (600 MHz, $CDCl_3$) δ 5.09 to 5.19 (m, 6H), 2.04 to 2.12 (m, 8H), 1.96 to 2.04 (m, 12H), 1.69 (s, 6H), 1.61 (m, 18H)

## Radiosynthesis of [3-$^{14}$C]-squalene (3)

**(6E,10E,14E,18E)-21-Bromo-2,6,10,15,19-pentamethylhenicosa-2,6,10,14,18-pentaene (13)**

A stirred solution of turbinaric acid **4** (700mg, 1.175mmol) in tetrahydrofuran (15ml) was treated with 1,1'-carbonyldiimidazole (370mg; 2.28mmol) in one portion. The mixture was heated at 60°C for 1 hour. The reaction was monitored by TLC (after esterification of a sample in methanol) on silica plates eluted with diethyl ether/n-pentane (3:7, v/v) (methyl ester: $R_f$ = 1). 2-Mercaptopyridine N-oxide sodium salt hydrate (440mg, 2.68mmol, 2.28eq.) and 4-dimethylaminopyridine (50mg, 0.41mmol, 0.35eq.) were added and the mixture was stirred at

60°C for 1 hour. Bromotrichloromethane (30ml) was added and the mixture was stirred at 115°C for 45 min. The reaction was monitored by TLC ($SiO_2$, diethyl ether/n-Pentane (3:7, v/v), product: $R_f$ = 1). After cooling to room temperature, the mixture was diluted with diethyl ether (30ml) and brine solution (50ml). The mixture was poured into a separating funnel and extracted three times with diethyl ether (60ml). The combined organic solution was dried over $MgSO_4$, filtered and concentrated under vacuum. The residue was purified by chromatography on a silica gel column eluted with diethyl ether/n-pentane (5:95, v/v) to afford **13** (250mg, 34%) as a yellowish oil. TLC ($SiO_2$, diethyl ether/n-pentane (5:95, v/v), $R_f$ = 0.95).
$^1$H-NMR (600 MHz, $CDCl_3$) δ 5.23 (m, 1H), 5.08 to 5.18 (m, 4H), 3.43 (t, J=7.5 Hz, 2H), 2.53 (t, J=7.5 Hz, 2H), 1.94 to 2.14 (m, 16H), 1.69 (s, 3H) 1.63 (s, 3H), 1.61 (m, 12H)

**[1-$^{14}$C]-(4E,8E,12E,16E)-4,8,13,17,21-Pentamethyldocosa-4,8,12,16,20-pentaenenitrile (14)**

A stirred suspension of [$^{14}$C]-Potassium cyanide (355MBq, 0.16mmol) in dimethylsulfoxide (5ml) was treated with a solution of **13** (250.00 mg; 0.57mmol; 4.72 eq.) in dimethylsulfoxide (10ml). The mixture was stirred at room temperature overnight. A brine solution (40ml) was added. The product was extracted three times with diethyl ether (60ml). The combined organic solution was dried over $MgSO_4$, filtered and concentrated under vacuum. The residue was purified by chromatography on a silica gel column eluted with diethyl ether/n-pentane (1:9, v/v) to afford **14** (199MBq, 58% yield) as a colorless oil. TLC ($SiO_2$, diethyl ether/n-pentane (1:9, v/v), $R_f$ = 0.85).

**[1-$^{14}$C]-(4E,8E,12E,16E)-4,8,13,17,21-Pentamethyldocosa-4,8,12,16,20-pentaenal (15)**

Compound **14** (199MBq, 0.098mmol) was dissolved in a mixture of ether (1ml) and n-pentane (10ml) under argon. The solution was cooled at -78°C. A solution of 1.0M diisobutylaluminium hydride in dichloromethane (0.5ml, 0.5mmol) was added dropwise. The mixture was stirred for 3 hours at -78°C. The reaction was monitored by TLC ($SiO_2$, diethyl ether/n-pentane (1:9, v/v), product $R_f$ = 0.6).The mixture was quenched with diethyl ether (20ml) then with a 0.5M aqueous solution of HCl (20ml). The mixture was warmed to room temperature and extracted three times with diethyl ether (30ml). The combined organic solution was dried over $MgSO_4$, filtered and concentrated under vacuum. The residue was purified by chromatography on a silica gel column eluted with diethyl ether/n-pentane (1:9, v/v) to afford **15** as a colorless oil, directly used in the next step. TLC ($SiO_2$, diethyl ether/n-pentane (1:9, v/v), $R_f$ = 0.6).

**[3-$^{14}$C]-(6E,10E,14E,18E)-2,6,10,15,19,23-Hexamethyltetracosa-2,6,10,14,18,22- hexaene (3)**

A stirred solution of isopropyltriphenylphosphonium bromide (2.8g, 7.3mmol) in anhydrous tetrahydrofuran (8ml) was treated dropwise with a 1.6M n-Butyllithium solution in hexane (4.6ml, 7.4mmol) at -78°C. During the addition, the color progressively turned to dark red. The mixture was warmed at room temperature then heated to reflux. A solution of **15** from the above experiment in anhydrous diethyl ether (2ml) was added all at once under reflux. After stirring at reflux for 20min, a saturated aqueous solution of $NH_4Cl$ was poured then the mixture was cooled to room temperature. The solution was extracted three times with diethyl ether (30ml). The combined organic layers were washed twice with a brine solution. The organic solution was dried over $MgSO_4$, filtered and concentrated under vacuum. The residue was purified by chromatography on a silica gel column eluted with n-pentane to afford 129.9MBq of **3** as a colorless oil (65% yield over 2 steps, 98,3% radiochemical purity determined by HPLC according to the above conditions). TLC ($SiO_2$, n-pentane $R_f$ = 0.18)

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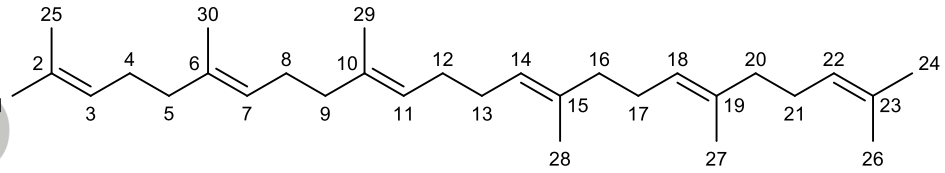
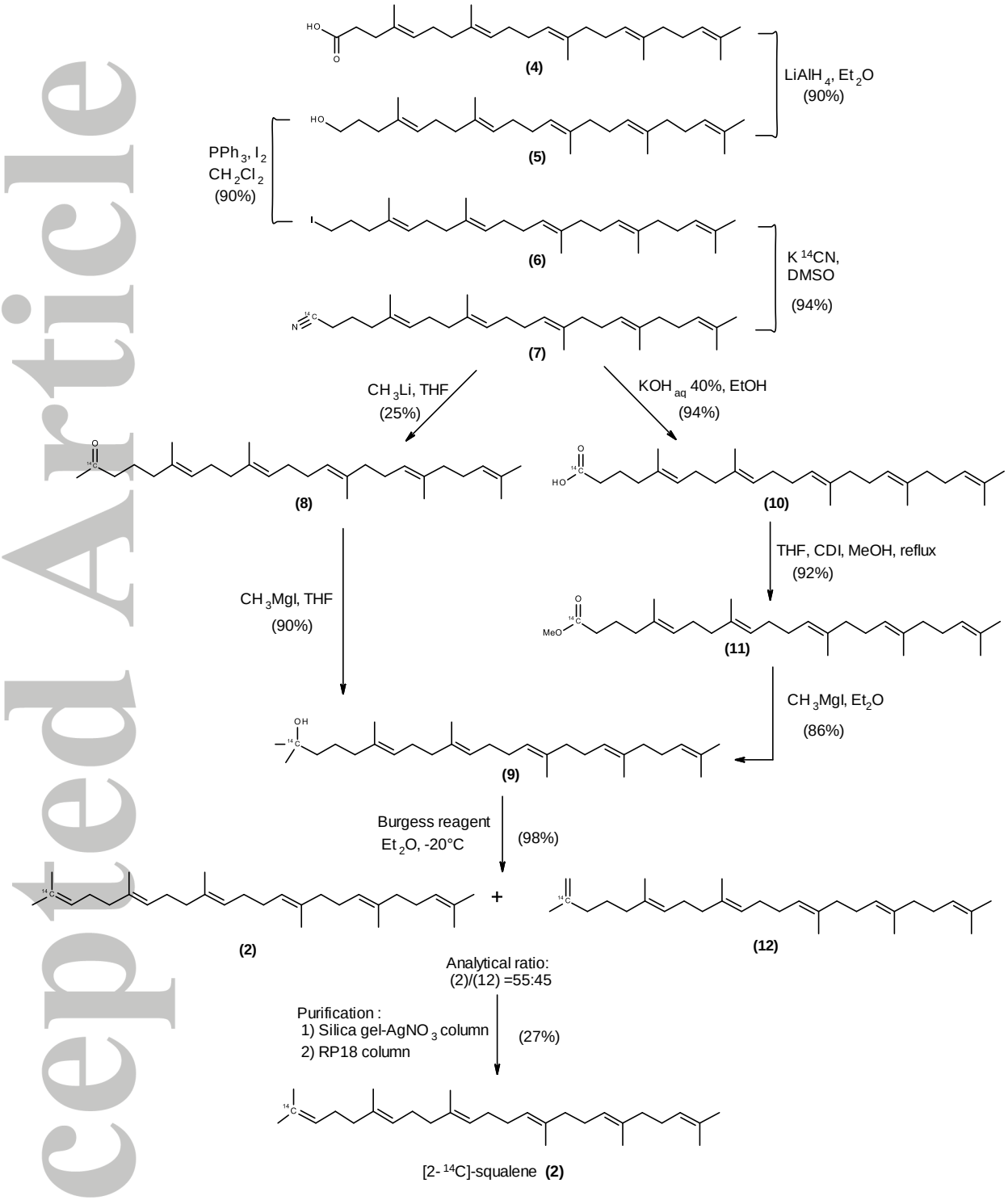
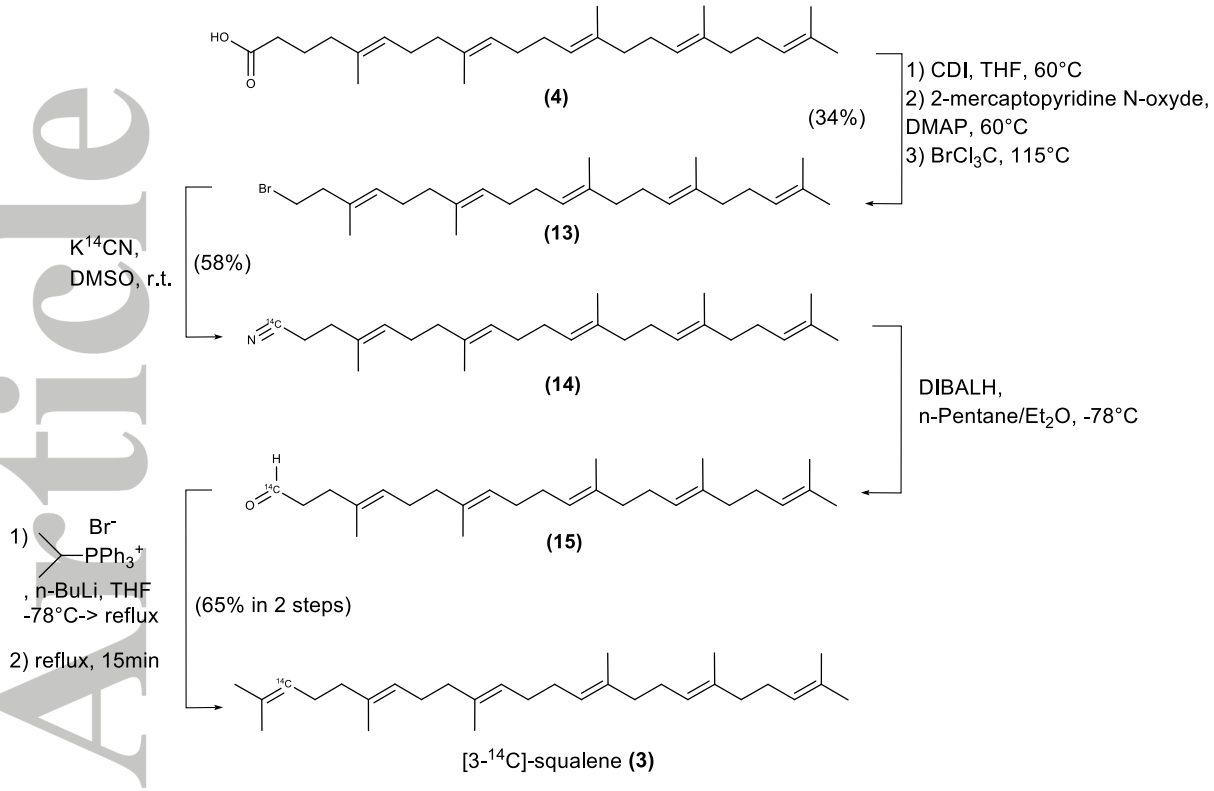



**Figure 1**. Structure of squalene **1**




**Scheme 1:** synthesis of [2-$^{14}$C]-squalene **2**




**Scheme 2:** synthesis of [3-$^{14}$C]-squalene **3**