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C-29 Tritiated ß-amyrin : Chemical synthesis aiming at the study of aromatization processes in sediments.

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Summary - Tritiated ß-amyrin (olean-12-en-3ß-ol, **2**) was synthesized in six steps, from epikatonic acid (**3**), with a specific activity of 550 GBq/mmole. The protracted incubation of this triterpene in a pond mud led by biodegradation to labelled aromatic tetracyclic hydrocarbons, identical with known sedimentary biomarkers.

Key Words : Triterpenes, tritiation, geochemistry, recent sediments, biological markers, biotransformation, aromatization.

One of the aims of organic geochemistry is to reconstruct the evolution of biological organic matter buried in sediments. This, first, requires the structural elucidation of the sedimentary organic constituents, which, then, can be related to their natural precursors. Among the lipidic biological markers, several tetra- and pentacyclic aromatic hydrocarbons related to C-3 oxygenated higher plant triterpenes have been identified in sediments and petroleums with a high terrigenous input (1-9). Their occurrence in recent sediments seemed to imply that they were formed by microbiological transformations taking place during early diagenesis of decaying higher plant material.

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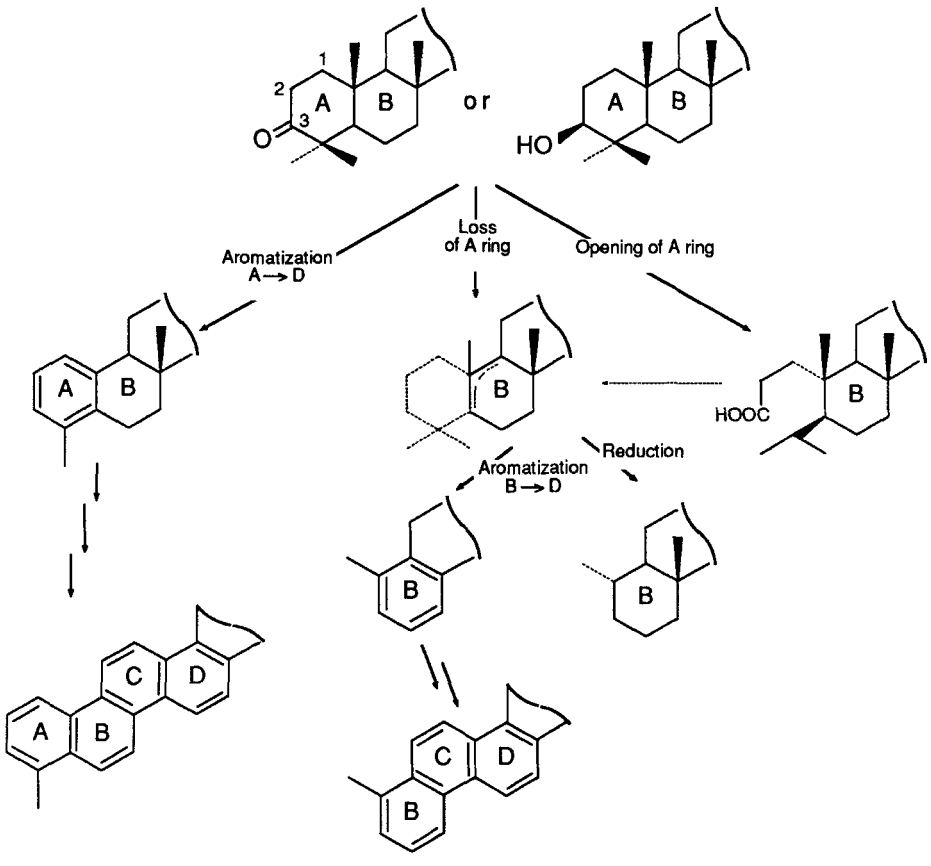
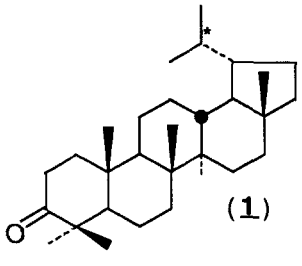


Figure 1:

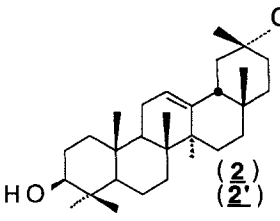
Hypothetical scheme of degradation of higher plant triterpenes in sediments

A hypothetical degradation pathway leading to their formation from plant triterpenoids (fig. 1) proposes that the aromatization starts with ring A, triggered by the elimination of the oxygenated function at C-3 and proceeds towards ring D (2,10). As an alternative ring A may be cleaved off and the remaining skeleton undergo progressive aromatization (3,4,11).



(1)

Conclusive characterization of triterpene related aromatic structures, as well as incubation of lupan-3-one 1 in recent muds have not only shown that the loss of ring A very probably resulted from microbial activity, but also that the aromatization processes seemed to occur easier with triterpenes containing a double bond in their skeleton (12). ß-amyrin 2 is such a molecule, known to occur in many



(2) R=H
(2') R=T

immature geological samples as a result of higher plant contribution . In this respect, it was also identified as a significant component in the recent subaquatic sediment which we have used in our experiments (13). In this publication, we describe the preparation of tritiated ß-amyrin (2) and the first results of its incubation within the subaquatic sediment. ß-Amyrin has been synthesized previously (14), but none of the chemical pathways described was adapted to the synthesis of an adequate radiolabelled compound. Indeed, the labelling of ß-amyrin must be performed in a chemical position which does not interfere with the aromatization processes. A high specific activity (~400 GBq/mmol) is also required *a*) to minimize the modification of concentration of natural ß-amyrin by introduction of synthetic labelled molecules in the sediment; *b*) to allow a sensitive monitoring of its degradation process.

Epikatonic acid (3ß-hydroxyolean-12-en-29-oic acid 3), a pentacyclic triterpene which possesses an acidic function at C-29 was therefore a suitable starting material. The acidic function could indeed lead by reduction with KBT_4 to the introduction of labelling at C-29 (KBT_4 has a higher specific activity than $NaBT_4$). Thus, we have developped a synthesis of labelled ß-amyrin starting from epikatonic acid (3), *via* a synthetic pathway as short as possible with labelled substances (fig. 2).

The carboxylic function at C-29 was protected as methyl ester with diazomethane (15) and the alcohol at C-3 as a *t*-butyldimethylsilyloxy ether (16). Reduction of the ester 5 allowed an easy introduction of the labelling. The reaction was first monitored with KBH_4; the addition of LiCl increased the yield of the reaction. The reduction with KBT_4 was achieved at the Service des Molécules Marquées (Centre d'Etudes Nucléaires, Saclay, France) and gave compound 6". Tosylation of 6 gave tosylate 7, quite unstable, which was rapidly reduced. The hindered neopentylic alcohol was not efficiently reduced with $LiAlH_4$, but superhydride $LiBHEt_3$ (17) gave 8 in good yield (66%). Finally, the deprotection of the alcohol at C-3 was performed with Bu_4NF (16).

The total yield of the six step synthesis was 35% for the cold synthesis but only 12% with the radioactive compounds. The difference in the two syntheses resides in the three steps achieved with labelled molecules. The decrease in the yield may stem from two differences : a higher dilution during reaction, and certainly some radiolysis during purification processes. The specific activity of the tritiated ß-amyrin was 550 GBq/mmole.

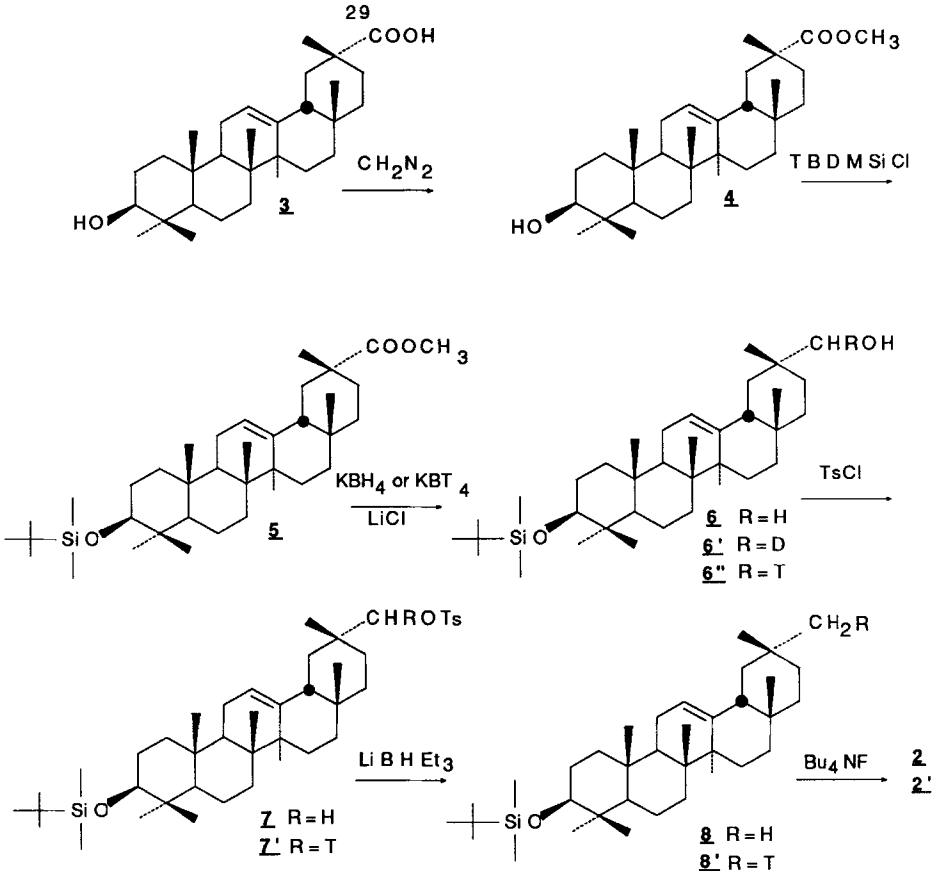


Figure 2 :

Synthetic scheme for the preparation of C-29 tritiated ß-amyrin **2'** from epikatonic acid **3**

In a large scale experiment undertaken to investigate the validity of the proposed degradation scheme (fig. 1), tritiated ß-amyrin was incubated in a recent anoxic mud collected from a pond of the Rhine valley (Huttenheim, France). The labelled compound was incubated for varying durations (from 15 days to one year or more) and under various conditions (in sterile conditions, or under inert atmosphere, or in conditions similar to the natural ones). The complete results of this exhaustive study will be presented in a further publication. We describe here only a punctual result which demonstrates the usefullness of tritiated ß-amyrin.

To 150 ml mud from a well characterized pond, 75 MBq of tritiated ß-amyrin were added and left in the dark at room temperature. After five months, the incubated sample was freeze-dried and the powdery sediment obtained was extracted twice with dichloromethane at

room temperature, and twice with chloroform at 50°C. The organic phases were pooled and the mixture was analyzed by successive TLC with adapted references. By this technique we were able to separate from the crude extract a non polar fraction which was further analyzed and separated by SiO_2 TLC into saturated, unsaturated and aromatic hydrocarbons. This fraction contained a major labelled compound (fig. 3) which was identified as diaromatic structure **9** (des-*A*-26,27-dinoroleana-5,7,9,11,13-pentaene) by co-elution with the corresponding reference compound (4,9) on SiO_2 TLC and on reverse phase HPLC (RP-18); furthermore, after reaction with bromine in presence of $FeCl_3$, the brominated labelled compounds co-eluted with those of the brominated reference (SiO_2 TLC, RP-18 HPLC).

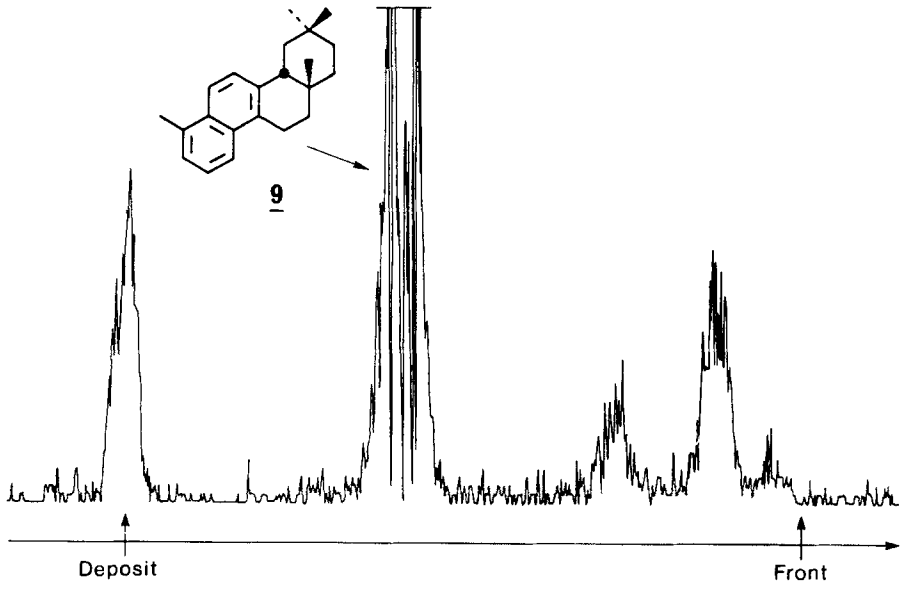


Figure 3 :

Radio TLC of the less polar fraction obtained from the mud sample after five months of incubation of labelled ß-amyrin

The compound **9** was not detected after incubation under sterile conditions. This result is thus indicative of a biological aromatization process and confirms one of the degradative pathways proposed in figure 1 : degradation of A ring followed by aromatization.

Experimental

Melting points were measured on a Reichert microscope and are uncorrected. $[\alpha]_D$ were measured on a Perkin-Elmer 141 polarimeter in $CHCl_3$. IR spectra were recorded in KBr on a

Pye Unicam SP3-3000S infrared spectrometer (Philips). ^{1}H NMR spectra were recorded on a Bruker SY (200 MHz) apparatus with $CHCl_3$ (7.27ppm) as internal standard. The chemical shifts are reported in ppm down field from TMS. MS were measured on a Thomson THN 208 B or a LKB 9000S apparatus by direct introduction using an ionization energy of 70 eV by electronic impact or chemical ionization (CH_4 orNH_3). Microanalyses were performed by the Strasbourg Division of the Service Central de Microanalyses of CNRS. TLC were run on pre-coated plates of silica gel $60F_{254}$ (Merck). Radioactivity on TLC plates was monitored with a Berthold TLC-Linear analyser LB. 2820. Radioactivity in solution was measured with an Intertechnique SL4000 liquid scintillation counter equipped with external standard and the samples were counted in the ACS (Amersham, Great Britain) liquid scintillation cocktail. Tetrahydrofuran, pyridine and diglyme were freshly distilled over lithium aluminium hydride before use.

3ß-Hydroxyolean-12-en-29-methyloate (4).

To a solution of diazomethane in ether (2 ml, 0.5 mmole/ml) **2** (10 mg, 0.022 mmole) in Et_2O (2 ml) was added . After 30 min at room temperature, the excess of diazomethane was eliminated by an argon flux until the yellow coloration disappeared. The ether was evaporated and the mixture was chromatographed on SiO_2 with CH_2Cl_2/Et_2O (1/1) as eluent. 9 mg of **4** (88%) were obtained.

$[\alpha]_D$=+33° (c = 0.5, $CHCl_3$).
IR : ν = 3620(m), 3455(w), 1725(m) cm^{-1}.
^{1}H NMR ($CDCl_3$) : δ = 0.80 (3H, s); 0.86 (3H, s); 0.94 (3H, s); 0.97 (3H, s), 1.00 (3H, s), 1.15 (3H, s); 1.21 (3H, s); 3.24 (1H, m, 3α-H); 3.66 (3H, s, $COOCH_3$); 5.23 (1H, dd, H-12, J≈3.5Hz).
MS (E.I.) : m/z = 470(M^+, 13%), 262(100), 207(14), 203(7), 189(8).
Anal. = Found C: 79.2 H: 10.8; $C_{31}H_{50}O_3$ requires C: 79.10 H: 10.71.

3ß-*t*-Butyldimethylsilyloxyolean-12-en-29-methyloate (5).

To a solution of **4** (7 mg, 0.015 mmole) and of imidazole (3 mg, 0.044 mmole) in DMF (1.5ml), cooled in an ice bath, a solution of *t*-butyldimethylsilyl chloride (4.5 mg, 0.030 mmole) in DMF (1 ml) was introduced dropwise . The mixture was stirred 2h at room temperature under Ar. CH_2Cl_2 was added and the organic phase was washed with water, with a saturated NaCl solution and dried with Na_2SO_4. The solvent was evaporated and the crude extract was chromatographed over silica gel (CH_2Cl_2/hexane 1/1) to yield 8 mg (92%) of **5**.

m.p. 138-138.5°C
^{1}H NMR ($CDCl_3$) : δ = 0.04 (6H, s, Me on Si); 0.76 (3H, s); 0.86 (3H, s), 0.89 (9H, s, tBu on Si); 0.91 (3H, s); 0.93 (3H, s); 0.96 (3H, s); 1.14 (3H, s); 1.21 (3H, s); 3.19 (1H, dd, 3α-H, J = 4.9 and 10.8 Hz); 3.67 (3H, s); 5.23 (1H, dd, H-12, J ≈ 3.3 Hz).
MS (C.I. CH_4) : m/z = 613 [($M + C_2H_5$)$^+$, 50%], 584(84), 569(72), 553(10), 527(100).

3ß-*t*-Butyldimethylsilyloxyolean-12-en-29-ol (6).

The reductive reagent was prepared by adding LiCl (1.5 mg, 0.03 mmole) to a suspension of KBH_4 (1.6 mg, 0.03 mmole) in diglyme (bis(2-methoxyethyl)ether, 2 ml). This

mixture was stirred during 30 min, then compound **5** (6 mg, 0.01 mmole) in diglyme (2 ml) was added dropwise at room temperature. The reaction mixture was refluxed for 24h under argon. After cooling to room temperature, the crude mixture was extracted with CH_2Cl_2. The organic phase was washed with water, saturated NaCl solution and dried with Na_2SO_4. Chromatography over silica gel (CH_2Cl_2/hexane 4/1) yielded 5 mg of a colourless oil (88%).

MS (E.I.) : m/z = 556 (M^+, 6%), 541(1), 526(4), 499(14), 321(3), 234(100), 190(21).

3β-*t*-Butyldimethylsilyloxy[29-^2H]olean-12-en-29-ol (6').

The reaction was carried out by the same procedure as for **6**, but with $NaBD_4$ instead of KBH_4 for the control of deuterium incorporation (yield 72%).

^1H NMR ($CDCl_3$) : δ = 0.04 (6H, s, Me on Si); 0.76 (3H, s); 0.86 (3H, s); 0.89 (9H, s, tBu on Si); 0.91 (3H, s); 0.94 (3H, s); 0.97 (3H, s); 1.14 (3H, s); 1.26 (3H, s); 3.20 (1H, dd, 3α-H, J = 4.9 and 10.9Hz); 5.21 (1H, dd, H-12, J ≈ 3.5Hz).
MS (E.I.) : m/z = 558 (M^+, 15%), 528(16), 501(30), 322(10), 236(100), 190(38).

3β-*t*-Butyldimethylsilyloxy[29-^3H]olean-12-en-29-ol (6").

The same procedure as for **6** was run on **5** (4 mg, 6.8 μmole) in the presence of KBT_4 (50 GBq, Specific Activity 2.2 TBq/mmole). The reaction had a slower evolution than with KBH_4. The yield was 70% after 72 h. Purification was carried out by TLC (CH_2Cl_2/hexane 4/1). 3 GBq of **6"** were obtained.

3β-*t*-butyldimethylsilyloxy-29-*p*-toluenesulfonyloleanen-12-ene (7).

The alcohol **6** (4.5 mg, 0.0081 mmole) was dried under vacuum, dissolved in a minimum amount of dry pyridine (2 ml) and an excess of p-toluenesulfonyl chloride (8 mg, 0.042 mmole, ~5 equiv) was added. After 3 h at room temperature, under argon, the reaction was quenched by addition of water. Extraction was performed with CH_2Cl_2; the organic phase was washed with water, with a 10% $NaHCO_3$ solution and dried with Na_2SO_4. Silica-gel chromatography (CH_2Cl_2/hexane 1/1) gave **7** (5 mg, 87%). This unstable compound must be kept under argon at -25°C.

^1H NMR($CDCl_3$) : δ = 0.05 (6H, s, Me on Si); 0.76 (3H, s); 0.83 (3H, s); 0.90 (9H, s, tBu on Si), 0.92 (3H, s); 0.93 (3H, s); 0.94 (3H, s); 1.07 (3H, s); 1.59 (3H, s); 2.46 (3H, s, Me on tosyl); 3.19 (1H, dd, 3α-H, J = 4.9 and 10.9Hz); 3.63 (2H, m, H-29), 5.17 (1H, dd, H-12); 7.35 (2H, m, H aromatic, J_{AB} = 8.1Hz); 7.78 (2H, m, H aromatic, J_{AB} = 8.1Hz)
MS (C.I. NH_3): m/z = 728[$(M+NH_4)^+$, 31%), 670(5), 596(6), 579(36), 557(14), 425(100), 407(31), 395(89), 393(23), 388(36), 234(48), 217(22), 216(45), 204(48), 91(87).

3β-*t*-Butyldimethylsilyloxy-29-*p*-toluenesulfonyl[29-^3H]olean-12-ene (7').

The reaction was carried out in the same conditions as for **7** but the yield was only 69%. This can be explained by the difficulty to maintain rigorously dry conditions with very small quantities.

3ß-t-Butyldimethylsilyloxyolean-12-ene (8).

To a solution of 7 (5 mg, 0.007 mmole) in dry THF (2 ml) was added a large excess of the superhydride $LiBHEt_3$ in THF (1 ml, 1 mmole/ml). The reaction mixture was refluxed under argon for 8h. Treatment with an aqueous solution of 10% NH_4Cl and extraction with CH_2Cl_2 furnished a crude product which was chromatographed on TLC (hexane) and yielded 2.5 mg (66%) of compound 8 (Rf = 0.94).

m.p. = 154-156°C
^1H NMR ($CDCl_3$) : δ = 0.04 (6H, s, Me on Si); 0.75 (3H, s); 0.83 (3H, s); 0.88 (3H, s); 0.89 (9H, s, tBu on Si); 0.91 (3H, s); 0.93 (3H, s); 0.96 (3H, s); 1.13 (3H, s); 1.26 (3H, s); 3.19 (1H, dd, 3α-H, J = 4.8 and 10.8Hz); 5.18 (1H, dd, H-12, J ≈ 3.5Hz).
MS (E.I.): m/z = 540 (M^+, 5%), 525 (2), 483 (27), 407 (10), 218 (100), 203 (39), 190 (30).

3ß-t-Butyldimethylsilyloxy[29-^{3}H]olean-12-ene (8').

The reaction was carried out in the same conditions as for 8, but during the reaction some solvent was evaporated and dry THF was added under argon to preserve the dry conditions. Even with such an adaptation, the yield was only 42%. Some starting material 7 was recovered (26%).

3ß-Hydroxyolean-12-ene (ß-Amyrin) 2.

A solution (3 ml) of tetrabutylammonium fluoride in THF (1 mmole/ml) was added to 5 mg (0.009 mmoles) of dry compound 8. The reaction mixture was stirred at room temperature for 12h. Then Na_2SO_4, 10 H_2O was added into the solution and the mixture was filtered and evaporated to dryness. The crude compound was chromatographed on TLC (CH_2Cl_2:Et_2O, 95:5, Rf 0.45) to give compound 2 (3.4 mg, 85 % yield).

^1H NMR ($CDCl_3$) : δ = 0.80 (3H, s); 0.84 (3H,s); 0.88 (6H, s); 0.94 (3H,s); 0.97 (3H,s); 1.00 (3H,s); 1.14 (3H,s); 3.23 (1H, dd, 3α-H, J ≈ 6 and 10 Hz); 5.17 (1H, dd, H-12, J ≈ 3.5 Hz).
MS (E.I.): m/z = 426(M^+, 7%); 411 (2), 218 (100), 207 (6), 203 (23), 189 (9).

3ß-Hydroxy[29-^{3}H]olean-12-en (^{3}H-ß Amyrin)(2').

The reaction was carried out in the same conditions as for 2; during the reaction, some solvent was evaporated under argon and dry THF was added to preserve the dry conditions. After 16h, the reaction was stopped with Na_2SO_4, 10 H_2O. The mixture was filtered and the tritiated compound was isolated on TLC (CH_2Cl_2) using synthetic non labelled ß-amyrin as reference. The yield was 74%. The concentration of a solution of pure ^{3}H-ß-amyrin was evaluated by a gas chromatographic analysis and the radioactive concentration was measured by scintillation counting. The specific activity of ß-amyrin deduced was 550 GBq/mmole.

Incubation technique

The mud was taken from the deep and always submerged part of a pond located

near Huttenheim (Alsace, France). It was fluid but heterogeneous and incubation was started on the same day. Tritiated ß-amyrin (57µg, 74 MBq) was dissolved in ethanol (1 ml with 0.5% Tween 80) and incorporated with shaking in 150 ml mud in a 250 ml flask. For sterile incubation, the mud was first sterilized (in three successive runs : 1.8b (117°C, 0.5 h) and two times 2.5b (128°C, 1 h). The ß-amyrin was added in sterile conditions. At the end of incubation the sterility was checked by platting in Hestrin and Schramm medium (18) and the mud samples were freeze-dried. The powder was extracted with 2x200 ml CH_2Cl_2 at room temperature and 2x200 ml $CHCl_3$ at 50°C. The extract was separated on TLC (eluent CH_2Cl_2). The less polar fraction was scraped off, reextracted and further separated on TLC (eluent hexane).

Bromination of 9

The reference compound and the labelled compound were mixed and dissolved in 5 ml of $CHCl_3$; 2 ml of a solution of Br_2 in acetic acid (1/1) was added and few drops of a 27% solution of $FeCl_3$. After 3h at room temperature, the mixture was extracted with hexane and the organic phase was washed with a saturated sodium sulfite solution and dried with Na_2SO_4. After evaporation the residue was analyzed by TLC (hexane/AcOEt 85/15).

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