Synthesis of β-Sitosterol-3-^{14}C

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SUMMARY

The synthesis of β-sitosterol-3-^{14}C, specific activity 5 mCi/mM, radiochemical purity ≥98 %, is described. Starting from stigmasterol, the complete synthesis consists of eleven reaction steps. It is based on methods described in the literature. For some of the steps, a considerable improvement in yield has been achieved. The actual labelling step is the condensation of 4-oxa-Δ^5-stigmasten-3-one with phenyl acetate-^{14}C in the presence of sodium hydride. β-Sitosterol-3-^{14}C has been obtained with a chemical yield of 23 %, based on 4-oxa-Δ^5-stigmasten-3-one. The radiochemical yield was 10.3 %. Chemical and radiochemical purities of β-sitosterol-3-^{14}C have been determined by means of thin-layer chromatography, gas-liquid chromatography and mass spectrometry.

Introduction.

For work in our institute on sterol metabolism of insects, in particular on the conversion of β-sitosterol into cholesterol and a possible inhibition of this dealkylation, β-sitosterol labelled with ^{14}C in the nucleus was needed. β-Sitosterol is a sterol widely occurring in plants; it differs from the zoosterol cholesterol in having an additional ethyl group attached to carbon atom 24 (Fig. 1). Plant-eating insects are able to remove this ethyl group. For these insects sitosterol is an important source of cholesterol, which in its turn is a precursor of the vital moulting hormone ecdysone.

A general method for labelling steroids with ^{14}C in ring A of the steroid nucleus was developed as early as 1947 by Turner [1, 2], who prepared cholestenone-3-^{14}C from cholestenone. The conversion of cholestenone into cholesterol has also been described [3, 4, 5]. An alternative route leading to ^{14}C substitution at the 4-position has been developed by Fujimoto [6, 7]. Though the method of Fujimoto gives higher radiochemical yields, we chose the method of Turner, as we had better access to labelled phenyl acetate and

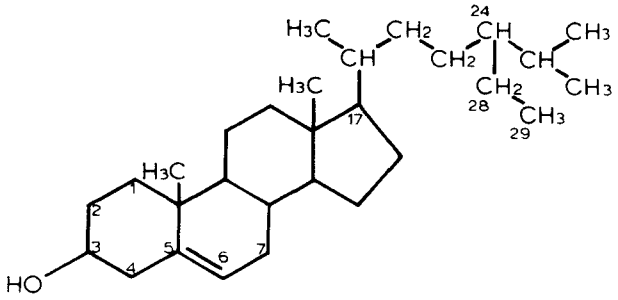
H3C
CH2
CH
CH2
24
CH
CH3
CH
CH3
CH2
28
CH3
29
H3C
17
H3C
1
2
3
4
5
6
7
HO
β-Sitosterol (Δ5-Stigmasten-3β-ol)

FIG. 1.

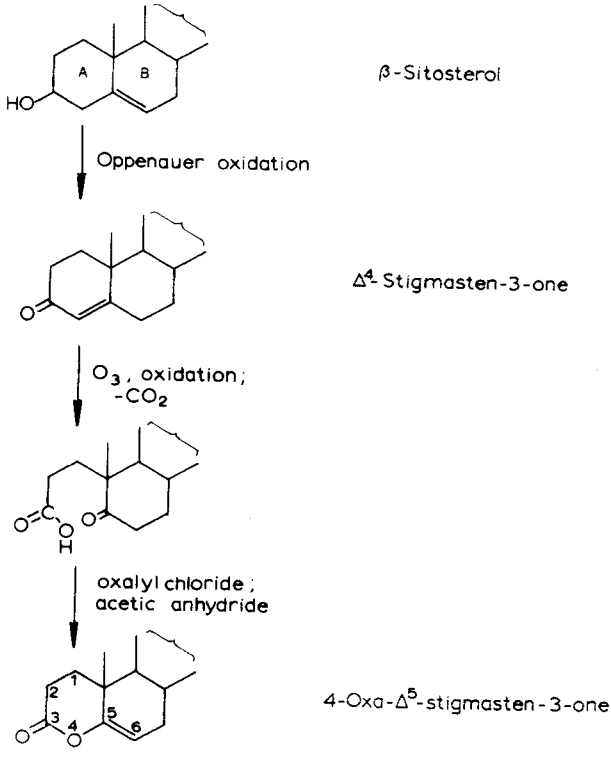
A
B
HO
β-Sitosterol
Oppenauer oxidation
Δ4-Stigmasten-3-one
O3, oxidation;
-CO2
oxalyl chloride;
acetic anhydride
4-Oxa-Δ5-stigmasten-3-one

FIG. 2.

preferred carrying out a synthesis with phenyl acetate instead of a Grignard reaction with a small amount of methyl-^{14}C-iodide.

A scheme of the reactions is given in Figures 2 to 4. Starting from pure β-sitosterol, the synthesis comprises seven steps, the net result being the replacement of carbon atoms 3 and 4 by the carbon atoms of acetic acid, labelled with ^{14}C. Commercial β-sitosterol could not be used as a starting material, since β-sitosterol isolated from plants contains large amounts of campesterol in percentages ranging up to 35 %. This homologue is difficult to separate from β-sitosterol on a preparative scale. Neither is it likely that its derivatives can be separated in the course of the synthesis. We therefore started with pure β-sitosterol prepared from stigmasterol, following the method of Steele and Mosettig [8], with slight modifications. By an Oppenauer oxidation (Fig. 2), the OH group is oxidized, while the double bond is shifted

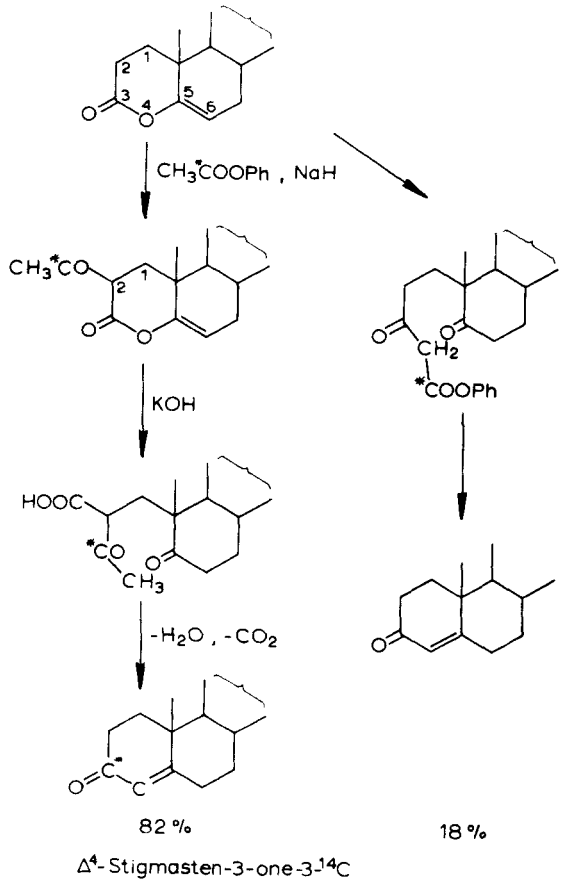

FIG. 3.

from ring B to ring A, in conjugation with the keto function [16]. Then with ozone, the double bond is attacked and the ring is opened. By further oxidation an acid is formed, which looses carbon dioxide and the keto acid shown is obtained. By treatment with oxalyl chloride and acetic anhydride, a method described by Fujimoto and Jacobson [7], the carboxyl group forms an anhydride with the enolized keto function. We have been able to raise the yield of the conversion of Δ^4-stigmasten-3-one into 4-oxa-Δ^5-stigmasten-3-one to 55 %. This enol lactone is the actual starting material for the radio-active synthesis (Fig. 3). It is condensed with ^{14}C-labelled phenyl acetate in the presence of sodium hydride. The $CH_3{}^{14}CO$ group is attached to carbon atom 2. By treatment with potassium hydroxide, the enol lactone is hydrolized, the ring is opened and by rotation around the C_1-C_2 bond as axis, the carbon atoms of the CH_3CO group come to occupy positions 3 and 4 in the steroid skeleton.

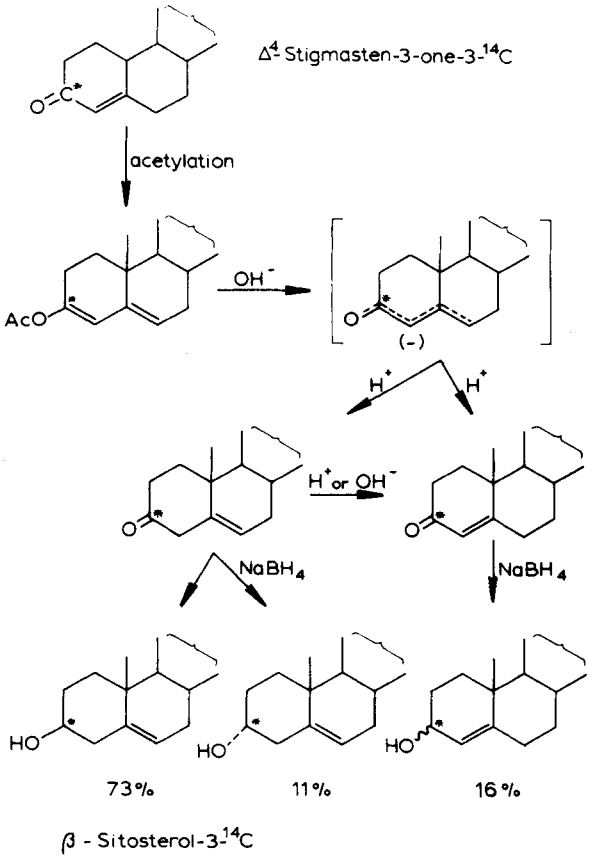

FIG. 4.

Water and carbon dioxide are split off and Δ^4-stigmasten-3-one-3-^{14}C is obtained. In a side reaction, the carbonyl function of the enol lactone condenses with the CH_3 group of the phenyl acetate. The resulting condensation product is also hydrolized and splits off water and carbon dioxide, thus forming unlabelled stigmastenone. The result is that the stigmastenone obtained has a somewhat lower specific activity than the phenyl acetate-^{14}C. We found a decrease of 18 %, which means that 82 % of the stigmastenone is formed by route I, and 18 % by route II. The stigmastenone was purified twice by column chromatography. The overall yield was 36 %, based on enol lactone; the radiochemical yield was 16 %.

Two more alterations are required for conversion of the Δ^4-3-keto steroid into β-sitosterol : shift of the double bond from Δ^4-position in ring A to Δ^5-position in ring B and reduction of the carbonyl function to a β-hydroxyl group. By treatment with isopropenyl acetate (Fig. 4), the enolized ketone is acetylated. For enolization a hydrogen atom at C-6 is preferred to a hydrogen atom at C-2. The acetoxy-$\Delta^{3,5}$-diene obtained is hydrolized; the intermediate anion takes up a proton from the solvent (methanol, ether, water); attachment of the proton to position 4 is kinetically preferred [10]. When the Δ^5-3-keto steroid formed is reduced, it yields β-sitosterol and the epimer with the hydroxyl group in axial orientation. Some Δ^4-3-keto steroid is also formed, either by direct attachment of a proton to position 6, or by isomerization of the Δ^5-keto steroid, which is known to occur very easily under the influence of acid or base. By reduction, two more isomers of β-sitosterol are formed.

The deconjugation of Δ^4-3-keto steroids has been the subject of a series of investigations [9, 10, 11, 12, 13, 14]. It is possible to obtain the intermediate anion directly from the Δ^4-3-ketone, e.g. by reaction with potassium *t*-butoxide [11]. However, the indirect route via the enol acetate is to be preferred from a preparative point of view.

The conversion of the enol acetate into β-sitosterol by reaction with sodium borohydride has been described by Fujimoto and Jacobson [7]. Following their procedure, we obtained predominantly the Δ^4-isomers of β-sitosterol. We have therefore studied the influence of reaction conditions more extensively and obtained best results when the hydrolysis was carried out at $-10°$ C, at a NaOH-concentration of about 5.10^{-3} mM/ml, in a solution containing sodium borohydride so that the ketone formed is reduced immediately. Under these conditions, the isomerization of the Δ^5- into the Δ^4-ketone is not yet important, while the enol acetate is hydrolized at moderate rate; the sodium borohydride is more stable than at a lower pH [15]. Progress of the reaction was followed by means of thin-layer chromatographic analysis. β-Sitosterol is formed in 73 % yield. After purifying the product twice by column chromatography, and by recrystallization, β-sitosterol-3-^{14}C is obtained in 64 % yield.

EXPERIMENTAL.

β-*Sitosterol.*

Stigmasterol (Fluka), which, as was found by gas chromatography, contained a trace of campesterol besides about 5 % β-sitosterol, was used for the synthesis of β-sitosterol. We have followed one of the two methods given by Steele and Mosettig [8], viz. the route via i-stigmasteryl methyl ether, since in this route the amounts are easier to handle than in the route via i-stigmasterol. The intermediates of the synthesis were thoroughly purified. β-Sitosterol, after purification by column chromatography, contained, besides a trace of campesterol, some stigmasterol and stigmastanol. No further attempt was made to remove stigmasterol and stigmastanol, because impurities resulting from these compounds could more easily be separated in some of the subsequent reaction steps. The various reaction steps of the synthesis of β-sitosterol were carried out in several batches. Their best results gave an overall yield of 47 %.

Δ^4-*Stigmasten-3-one.*

β-Sitosterol was oxidized by the Oppenauer method as described by Barton and Jones [16]. The yield after purification by column chromatography and recrystallization from aqueous methanol was 71 %.

4-*Oxa-*Δ^5-*stigmasten-3-one.*

The procedure given by Fujimoto and Jacobson [7] has been slightly modified.

Δ^4-Stigmasten-3-one (2.4 g, 5.86 mM) was dissolved in ethyl acetate (30 ml) and glacial acetic acid (30 ml), and ozonized at −10° C until the initially formed yellow colour had disappeared. When water (10 ml) and hydrogen peroxide (30 %, 1 ml) were added, a white crystalline precipitate was formed. After the mixture had been standing for 18 hours at 20° C, it was cooled to 0° C. The crystals (A) were then separated from the mother liquor (B), and dissolved in a mixture of hydrogen peroxide (30 %, 1.5 ml), ethyl acetate (65 ml) and acetic acid (5 ml). Efficient stirring for 80 hours was required. The solution was taken to dryness in vacuo; traces of water were removed by azeotropic distillation with benzene (during warming up, a gas was evolved); traces of benzene were removed in vacuo. Then the residue was dissolved in freshly (!) distilled oxalyl chloride (6 ml) by stirring at −2° C, under nitrogen. After the solution had been standing at 0° C for 70 hours, it was taken to dryness in vacuo. The residue was refluxed with acetic anhydride (50 ml) for 2.5 hours, after which the solution was concentrated in vacuo and 4-oxa-Δ^5-stigmasten-3-one crystallized. It was dried over sodium

hydroxide and finally sublimed at 150° C and 10^{-3} mm Hg. The yield was 610 mg, m. p. 106.5-107° C (lit. [7] m. p. 103-107° C).

The mother liquor (B) was also taken to dryness in vacuo; traces of water were removed by azeotropic distillation with benzene. The colourless only residue was treated with oxalyl chloride and acetic anhydride as described above, yielding a second crop of 4-oxa-Δ^5-stigmasten-3-one (730 mg, m. p. 103-105° C). The total yield was 1.34 g (3.23 mM, 55 %). This moisture-sensitive compound was stored in vacuum sealed ampoules.

Δ^4-Stigmasten-3-one-3-^{14}C.

A solution of phenyl acetate-^{14}C (2.8 mM, 380 mg; spec. act. 6.28 mCi/mM) in benzene (2 ml) was stirred with 100 mg sodium hydride for 15 minutes, shortly before use, in order to remove traces of moisture. The clear solution was separated from the sodium hydride by means of a pipette. In a 50 ml flask, provided with a magnetic stirrer and connected to a gas burette, a mixture of 4-oxa-Δ^5-stigmasten-3-one (1.5 mM, 620 mg), finely powdered sodium hydride (875 mg), the benzene solution of phenyl acetate-^{14}C and 10 ml of dry benzene, were stirred very well at room temperature until no more hydrogen was evolved (70 hours). Excess sodium hydride was decomposed by adding 1 ml of concentrated hydrochloric acid. The mixture was extracted with ether, the ether solution washed twice with water, dried over sodium sulphate and evaporated. Volatile components such as phenol, phenyl acetoacetate and phenyl acetate, were removed by heating at 80° C in vacuo (10^{-3} mm Hg). The residue was dissolved in methanol (55 ml) and water (20 ml), containing potassium hydroxide (700 mg). This solution was refluxed for 18 hours by heating in an oil bath at 85-90° C. After addition of acetic acid (2 ml), the solution was concentrated to a volume of 5 ml and extracted with ether. The ether solution was washed with water and evaporated to dryness in vacuo. The residue was dissolved in n-hexane/benzene (1 : 1) and purified by column chromatography on florisil. The column was eluted with n-hexane/benzene mixtures, varying the benzene concentration from 0 to 100 % in steps of 10 % after each 100 ml. The fractions containing Δ^4-stigmastenone-3-^{14}C were collected and the purification repeated. The yield was 222 mg = 0.54 mM, i.e. 36 % based on 4-oxa-Δ^5-stigmasten-3-one.

3-Acetoxy-$\Delta^{3,5}$-stigmastadiene-3-^{14}C.

Δ^4-Stigmasten-3-one-3-^{14}C, (0.5 mM, 207 mg), was dissolved in isopropenyl acetate (40 ml), and refluxed for 2.5 hours after addition of 0.04 ml of concentrated sulphuric acid (*d* 1.84). The mixture was concentrated to half its volume, isopropenyl acetate (20 ml) was added and it was refluxed for 3 hours. Then once again the mixture was concentrated to half its volume,

isopropenyl acetate (20 ml) added and refluxed for 3 hours. Finally it was taken to dryness in vacuo.

β-Sitosterol-3-^{14}C.

The 3-acetoxy-$\Delta^{3,5}$-stigmastadiene-3-^{14}C was dissolved in a mixture of ether (100 ml) and methanol (75 ml); a solution of sodium hydroxide (90 mg = 2.3 mM; i.e. 0.8 mM in excess of the amount needed for neutralizing the sulphuric acid used in the preparation of the enol-acetate) in water (8 ml) was added. After cooling at -10^{o} C, a total of 630 mg sodium borohydride was added in the course of 100 hours, in portions of 70 mg, each dissolved in 10 ml of methanol/water (60 : 40). The progress of the reaction was followed by thin-layer chromatographic analysis. After 140 hours, conversion was complete. The temperature was raised to 5^{o} C and excess sodium borohydride decomposed by addition of concentrated hydrochloric acid (6 ml; *d* 1.18). The solution was boiled for 45 minutes, concentrated to 60 ml and extracted with ether (5 × 20 ml). The ether solution was washed with water (20 ml), dried over sodium sulphate and taken to dryness. The residue was dissolved in benzene (10 ml), n-hexane (30 ml) was added and it was purified by column chromatography on deactivated alumina (15 g; diameter of column 1 cm; for deactivation a mixture of 250 g Al_2O_3, according to Brockmann, and 14 ml of 10 % acetic acid was agitated for 2 days).

The column was eluted subsequently with 150 ml of n-hexane, 150 ml of n-hexane/ether (97 : 3), 100 ml of n-hexane/ether (94 : 6), 100 ml of n-hexane/ether (91 : 9) and 100 ml of n-hexane/ether (88 : 12). The eluent was collected in fractions of 50 ml. Each fraction was analyzed by thin-layer chromatography. The fractions containing β-sitosterol-3-^{14}C were combined and purification by column chromatography repeated. Finally, the β-sitosterol-3-^{14}C was recrystallized from methanol/water (9 : 1). The yield was 130.6 mg, 0.32 mM, i.e. 64 % based on Δ^{4}-stigmasten-3-one-3-^{14}C. The product was dissolved in benzene/toluene (13 : 1) and stored in vacuum-sealed ampoules.

Specific activity.

A sample of the β-sitosterol-3-^{14}C benzene/toluene solution was taken to dryness and the residue sublimed in high vacuum, in order to remove traces of solvent. A solution of an accurately known β-sitosterol-3-^{14}C concentration was prepared in a liquid scintillator, to which carrier β-sitosterol was added (about 0.5 mg/ml). The composition of the liquid scintillator was 5 g PPO and 0.5 g POPOP in 1 l toluene. Activity measurements were carried out with 15 ml solution in a Packard Tri-Carb liquid scintillation spectrometer, model 3375. The specific activity found was : 5.17 ± 0.03 mCi/mM.

Thin-layer chromatography.

Analyses were carried out on silica gel (silica gel H, Merck, layer thickness 0.25 mm; activation of layer by heating for one hour at 110° C) in toluene/ethyl acetate (3 : 1). Detection under UV light ($\lambda = 366$ nm) after spraying with 10 % H_2SO_4 and heating at 150° C for 15 min. The following R_f values were found : β-sitosterol 0.36; Δ^5-stigmasten-3 α-ol 0.45; Δ^4-stigmasten-3 β-ol 0.42; Δ^4-stigmasten-3 α-ol 0.49; campesterol 0.36. In toluene/ethyl acetate (4 : 1), the following R_f values were found : β-sitosterol 0.26; Δ^4-stigmasten-3 β-ol 0.34; $\Delta^{3,5}$-stigmastadiene 0.7-0.8; Δ^4-stigmasten-3-one 0.50; campesterol 0.26. Autoradiograms on Kodak no screen X-ray film and a radioscan of β-sitosterol-3-^{14}C are shown in Figure 5. Judging from the densities on the film,

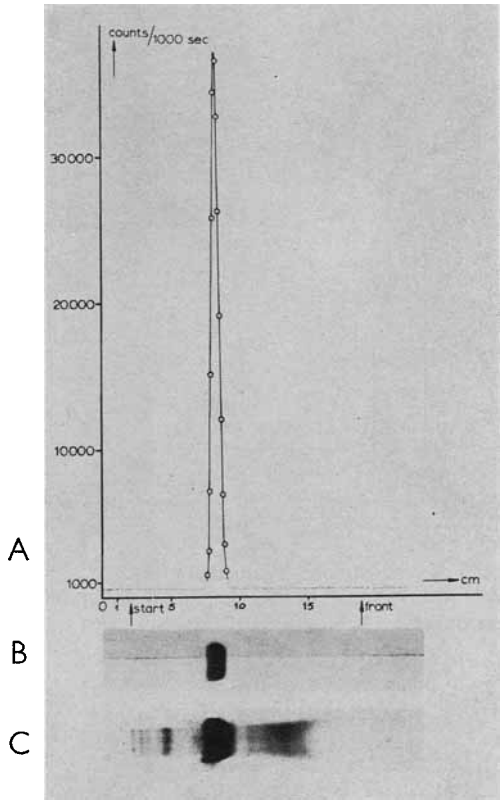

FIG. 5. Thin layer chromatogram of β-sitosterol-3-^{14}C silicagel H, toluene/ethyl acetate 3 : 1.
A. scan of chromatogram, in steps of 1 mm,
B. autoradiogram, exposure time 0.7 h,
C. autoradiogram, exposure time 70 h.
The main impurities are estimated at < 0.1 %.

even the main impurities are present in amounts much less than 1 %, probably about 0.1 %. This is confirmed by the radio-scan : one sharp peak at the R_f for β-sitosterol, and virtually no activity elsewhere.

Gas chromatography.

A gas chromatographic analysis of β-sitosterol-3-^{14}C methyl ether, according to the method described by Clayton [17], was carried out. Fractions were collected in glass tubes by cooling the gas stream emerging from the column. The radioactivity of each fraction was determined after the glass tubes had been washed with 15 ml scintillation liquid, containing 10 mg cholesterol, 5 g PPO and 0.5 g POPOP per 1 l toluene. The radio-histogram together with the mass peaks are shown in Figure 6. Campesterol is present in about 0.5 %.

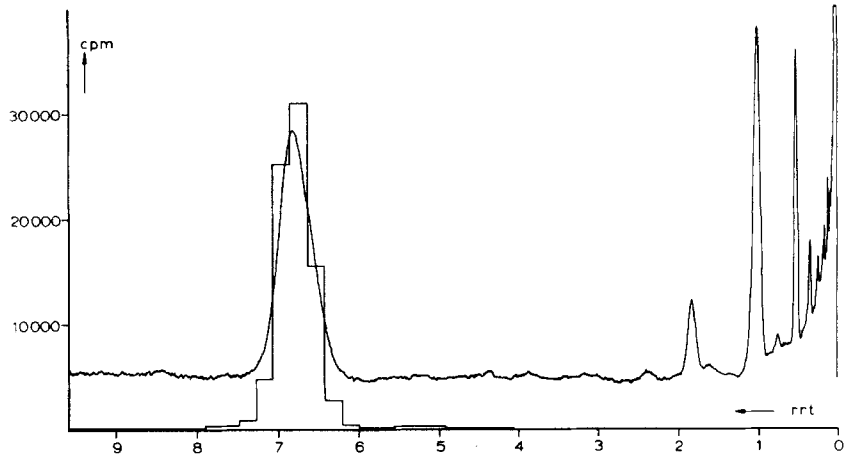

FIG. 6. Gaschromatogram of β-sitosterol-3-^{14}C methyl ether.
The mass peaks with r.r.t. < 2 are artefacts of the methylation. The histogram shows the activities of separate fractions. Radiochemical impurity at r.r.t. 5.3 : < 0.5 % (campesterol methyl ether).

Mass spectrometry.

A mass spectrum was run on a Hitachi-Perkin Elmer RMU-6E, with an electron energy of 70 eV. Part of the spectrum and the interpretation are shown in Table 1. Campesterol might be present in circa 0.7 %; the presence of about 1 % of other molecules with M.W. 416 cannot be excluded.

TABLE 1. Mass spectrum of β-sitosterol-3-^{14}C.

M/e	Relative peak-height ± S.D. (1)	Molecular ion peaks of β-sitosterol $C_{29}H_{50}O$ (2)	$^{14}C\,C_{28}H_{50}O$ (2)	Fragment ion peaks (β-sitosterol-H_2O) (3)	Fragment ion peaks (β-sitosterol -CH_3) (4)	Molecular ion peaks of campesterol (5)
418	0.5 ± 0.1		P(M′ + 2) 0.5			
417	3.7 ± 0.2	P(M + 3) 0.6	P(M′ + 1) 3.2			
416	15.5 ± 0.2	P(M + 2) 5.2	P(M′) 10.3			
415	31.9 ± 0.5	P(M + 1) 32.2				
414	100	P(M) 100				
413	2.0 ± 0.1					
412	1.8 ± 0.1					
402	1.0 ± 0.1				P(M‴ + 3) 0.9	
401	4.0 ± 0.1				P(M‴ + 2) 3.8	P(M^{IV} + 1) 0.2
400	8.6 ± 0.2			P(M″ + 4) 0.2	P(M‴ + 1) 7.7	P(M^{IV} + 1) 0.7
399	25.5 ± 1.0			P(M″ + 3) 1.2	P(M‴) 24.3	
398	5.6 ± 0.2			P(M″ + 2) 5.2		
397	11.1 ± 0.4			P(M″ + 1) 10.8		
396	33.6 ± 1.4			P(M″) 33.6		
395	1.5 ± 0.1					

(1) Mean of 5 spectra, electron energy 70 eV.

(2) P(M′) = P(416) — P(M + 2) = 10.3. Calculated contribution of ^{14}C, based on spec. act. 5.17 mC/mmole and theoretical max. spec. act. 62.2 mCi/mmole : C/100-C = 9.2 %. Conclusion : presence of other molecules with M.W. 416 can not be excluded (stigmastanol?).

(3) P(M″) = P(396); for calculation of the contribution to peaks 397 to 400, the ratios found in the group of peaks 414 to 418 were used.

(4) P(M‴) = P(399) — P(M″ + 3); $C_{28}H_{47}O$.

(5) P(M^{IV}) = P(400) — P(M‴ + 1) — P(M″ + 4); campesterol circa 0.7 %.

DISCUSSION.

Chemical and radiochemical purities have been determined by means of thin-layer chromatography, gas-liquid chromatography and mass spectrometry. Thin-layer radio-chromatography showed that a few minor, unidentified impurities are present in amounts of about 0.1 %. By gas-liquid radio-chromatography the presence of 0.5 % campesterol was shown. The mass spectrum shows two values of interest :

(1) the contribution of campesterol is about 0.7 %, which is in excellent agreement with the value obtained from the radio gas-chromatogram;
(2) the peak intensity at M/e = 416 indicates the presence of about 1 % of an impurity of molecular weight 416, which is not present in pure β-sitosterol *.

We have not been able to identify this impurity, but vapour-programmed thin-layer chromatography [18] and TLC on argentated silica gel showed that, if β-sitosterol-3-^{14}C contains any 5 α-stigmastanol, it must be less than 1 %.

From these analytical results we may conclude that the β-sitosterol-3-^{14}C obtained, has a radiochemical purity of $\geqslant$98 %. Though we have paid much attention to the purification of intermediates, a trace of campesterol present in the starting material, could not be removed in the course of the synthesis; it therefore appears in the final product.

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