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INCORPORATION OF STEREOSPECIFICALLY LABELLED MEVALONIC ACID INTO PORIFERASTEROL BY *OCHROMONAS MALHAMENSIS*

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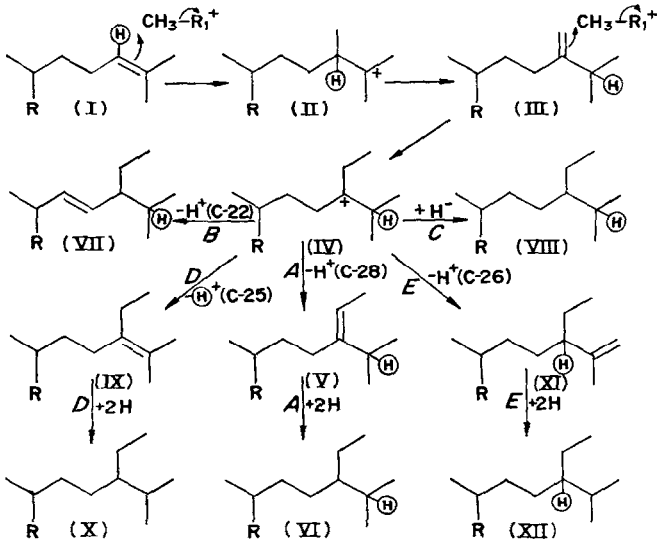
Key Word Index—*Ochromonas malhamensis*; Alga; phytosterol biosynthesis; poriferasterol; incorporation of labelled mevalonate; mechanism of biosynthesis.

Abstract—The use of [2-^{14}C, (4*R*)-4-$^{3}H_1$]-mevalonic acid has shown that the C-17, C-20 and C-25 hydrogen atoms of poriferasterol biosynthesized by *Ochromonas malhamensis* are derived from the 4-pro*R* hydrogen of mevalonic acid. The presence of tritium at C-25 is consistent with the involvement of a 24-ethylidene sterol in the C-24 alkylation mechanism in this organism.

INTRODUCTION

MANY studies on phytosterol† biosynthesis have been directed at the elucidation of the C-24 alkylation mechanism.[1,2] The C-24 alkyl groups of phytosterols arise by transmethyl-



SCHEME 1.

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† Common names of sterols used in this report: lanosterol, 4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol; cycloartenol, 4,4,14α-trimethyl-9β,19-*cyclo*-5α-cholest-24-en-3β-ol; 24-methylenecycloartanol, 4,4,14α-trimethyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol; obtusifoliol, 4α,14α-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol; stigmasterol, stigmasta-5,22-dien-3β-ol; poriferasterol, (24*R*)-24-ethylcholesta-5,22-dien-3β-ol; cholesterol cholest-5-en-3β-ol.

[1] E. LEDERER, *Biochem. J.* **93**, 449 (1964); *Quart Rev.* **23**, 453 (1969).

[2] L. J. GOAD and T. W. GOODWIN, in *Progress in Phytochemistry*, Academic Press, New York (1972).

ation reactions with methionine as the methyl donor.[1,2] One mechanism[3] for the elaboration of C_{29} sterols is indicated by route *A* in Scheme 1. Results demonstrating the utilization of Δ^{24} (I)[4] and 24-methylene (III)[5] sterols as substrates for alkylation and the observed hydrogen migration from C-24 to C-25 during 24-methylene (III)[6] and 24-ethylidene (V)[7,8] sterol production are consistent with route *A*. Moreover experiments using deuterium labelled methionine have indicated that a 24-ethylidene precursor (V) is produced and subsequently reduced to give a C_{29} sterol in the Chrysophyte *Ochromonas malhamensis* since only four deuterium atoms were incorporated into poriferasterol (XIX) by this organism.[9] However, incubations of CD_3–methionine with a slime mould[10] and with *Chlorella* species[11,12] have given in each case five deuterium atoms in the C_{29} sterols produced. This apparently indicates that 24-ethylidene sterols (V) are not formed in these organisms and routes *B*, *C*, *D* and *E* are possible alternatives from carbonium ion (IV).[10–12] Scheme 1 reveals that the location of the C-24 hydrogen atom of the precursor Δ^{24} sterol (I) in the product C–24 ethyl sterol (VI, VII, VIII, X, XII) will depend upon which route is employed. Evidence presented for the loss of this hydrogen in the case of stigmasterol production in tobacco and *Dioscorea tokoro* tissue cultures [13] implicates route *D* in these plant tissues while route *E* has been demonstrated[14] for sterol (XI) formation in leaves of *Clerodendrum campbellii*. The present work* shows that in *O. malhamensis* hydrogen migration from C-24 to C-25 occurs during poriferasterol (XIX) synthesis and provides further evidence for the operation of route *A* in this organism. Evidence for the postulated hydrogen migrations during squalene cyclization to give cycloartenol is also presented.

RESULTS AND DISCUSSION

The incubation of [2-^{14}C, (4*R*)-4-$^{3}H_1$]-mevalonic acid (XIII) with a rat liver preparation gives squalene-2,3-oxide (XIV), lanosterol (XVI) and cholesterol (XVII) labelled as shown in Scheme 2.[15–17] In higher plants and algae, including *O. malhamensis*, cycloartenol (XVIII) replaces lanosterol (XVI) as the sterol precursor[2,5,18,19] and this compound should be labelled from [2-^{14}C, (4*R*)-4-$^{3}H_1$]-mevalonic acid (XIII) as indicated.[20] In particular the cycloartenol (XVIII) will contain a tritium atom at C-24 and this fact permits the use of

* Part of the work submitted by A.R.H.S. in fulfilment of the requirements for the award of Ph.D., University of Liverpool, 1969.

[3] M. CASTLE, G. BLONDIN and W. R. NES, *J. Am. Chem. Soc.* **85,** 3306 (1963).
[4] P. T. RUSSELL, R. T. VAN ALLER and W. R. NES, *J. Biol. Chem.* **242,** 5802 (1967).
[5] J. R. LENTON, J. HALL, A. R. H. SMITH, E. L. GHISALBERTI, H. H. REES, L. J. GOAD and T. W. GOODWIN, *Arch. Biochem. Biophys.* **143,** 664 (1971).
[6] M. AKHTAR, P. F. HUNT and M. A. PARVEZ, *Biochem. J.* **103,** 616 (1967).
[7] K. H. RAAB, N. J. DE SOUZA and W. R. NES, *Biochem. Biophys. Acta* **152,** 742 (1968).
[8] L. J. GOAD and T. W. GOODWIN, *Europ. J. Biochem.* **7,** 502 (1969).
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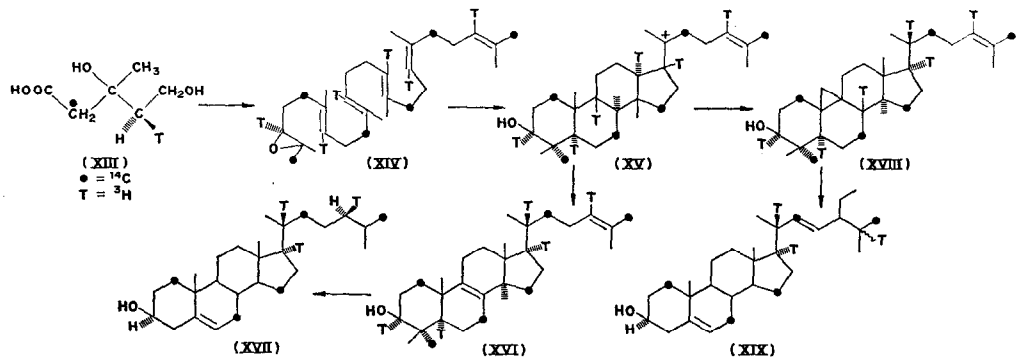
[10] M. LENFANT, R. ELLOUZ, B. C. DAS, E. ZISSMAN and E. LEDERER, *Europ. J. Biochem.* **7,** 159 (1969).
[11] Y. TOMITA, A. UOMORI and H. MINATO, *Phytochem.* **9,** 555 (1970).
[12] Y. TOMITA, A. UOMORI and E. SAKURAI, *Phytochem.* **10,** 573 (1971).
[13] Y. TOMITA and A. UOMORI, *Chem. Commun.* 1416 (1970).
[14] L. M. BOLGER, H. H. REES, E. L. GHISALBERTI, L. J. GOAD and T. W. GOODWIN, *Biochem. J.* **118,** 197 (1970).
[15] G. POPJÁK and J. W. CORNFORTH, *Biochem. J.* **101,** 553 (1966).
[16] J. W. CORNFORTH, R. H. DORNFORTH, C. CONNINGER, G. POPJÁK, Y. SHIMIZU, S. ICHII, E. FORCHIELLE and E. CASPI, *J. Am. Chem. Soc.* **87,** 3224 (1965).
[17] L. J. MULHEIRN and E. CASPI, *J. Biol. Chem.* **246,** 3948 (1971).
[18] L. J. GOAD, in *Biochemistry Society Symposium No.* 29. (edited by T. W. GOODWIN), p. 45, Academic Press, New York (1970).
[19] P. BENVENISTE, L. HIRTH and G. OURISSON, *Phytochem.* **5,** 45 (1966).
[20] H. H. REES, L. J. GOAD and T. W. GOODWIN, *Biochem. J.* **107,** 417 (1968).

[2-^{14}C, (4*R*)-4-$^{3}H_1$] mevalonic acid to determine the fate of the C-24 hydrogen atom of cycloartenol (XVIII) during its conversion into poriferasterol (XIX) by *O. malhamensis*.



SCHEME 2.

The 4,4-dimethyl sterol fraction isolated from a culture of *O. malhamensis* grown in the presence of [2-^{14}C, (4*R*)-4-$^{3}H_1$]-mevalonic acid was mixed with authentic cycloartenol and 24-methylenecycloartanol and the mixture separated after acetylation. Only about 5% of the initial radioactivity of the fraction was recovered in cycloartenyl acetate and 24-methylenecycloartanyl acetate, the remainder was associated with unidentified polyisoprenoid compounds. The specific radioactivities of both acetates remained constant during recrystallization confirming that mevalonic acid was incorporated into both cycloartenol and 24-methylenecycloartanol. Moreover both compounds had ^{3}H:^{14}C atomic ratios close to 1:1 as previously observed for these compounds biosynthesized from [2-^{14}C, (4*R*)-4-$^{3}H_1$]-MVA by other plant tissues[8,20] and in accord with the labelling pattern shown in Scheme 2.

TABLE 1. ^{3}H:^{14}C RATIOS OF PORIFERASTEROL AND DERIVATIVES FORMED FROM LABELLED MEVALONATE

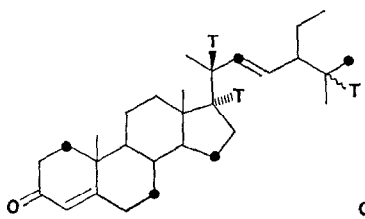
	^{3}H:^{14}C ratio	^{3}H:^{14}C atomic ratio	Theoretical ^{3}H:^{14}C atomic ratio
Poriferasterol (XIX) Sample 1	6·23	3·08:5	3:5
24-Ethylcholesta-4,22-dien-3-one (XX)	6·20	3·06:5	3:5
3-Oxobisnorchol-4-en-22-al (XXI)	5·30	2·10:4	1:2
22-(*N*-piperidyl)-bisnorchola-4,20(22)-dien-3-one (XXII)	3·65	1·40:4	1:4
Progesterone (XXIII)	3·68	1·09:3	1:3
Progesterone (XXIII) after equilibration	0·75	0·22:3	0:3
Poriferasterol (XIX) Sample 2	5·95	2·95:5	3:5
2-Ethyl-3-methylbutanal (XXIV)	11·40	1·12:1	1:1
2-Ethyl-3-methylbutanal (XXIV) after equilibration	10·10	1·00:1	1:1

The poriferasterol (XIX) obtained from an incubation of *O. malhamensis* with [2-^{14}C, (4*R*)-4-$^{3}H_1$]-mevalonic acid (XIII) had a ^{3}H:^{14}C atomic ratio of 3:5 (Table 1) as previously found in cholesterol (XVII)[16] and other phytosterols.[8,20,21] During the conversion of cycloartenol (XVIII) into poriferasterol (XIX) the 5α-tritium atom is lost during introduction of the Δ^5 bond whilst the 8β-tritium atom is presumably lost as a consequence of the opening of the 9β,19-cyclopropane ring to give a Δ^8 bond in a phytosterol precursor such as obtusi-

[21] H. H. REES, E. I. MERCER and T. W. GOODWIN, *Biochem. J.* **99**, 726 (1966).

foliol.[22] Oxidation of the labelled poriferasterol gave 24-ethylcholesta-4,22-dien-3-one (XX) with an unchanged $^3H:^{14}C$ atomic ratio (Table 1). This result demonstrated the absence of tritium at C-3 and showed that the 3α-tritium atom of cycloartenol (XVIII) was exchanged during conversion into poriferasterol (XIX). The same observation has previously been made in cholesterol (XVII) biosynthesis by animal tissues[16] and is explained by the involvement of 3-oxosteroids in the C-4 demethylation process.[23]

The loss of one ^{14}C atom (C-26) accompanied by one tritium atom upon ozonolysis of the 24-ethylcholesta-4,22-dien-3-one (XX) to give 3-oxo-bisnorchol-4-en-22-al (XXI) demonstrated the localization of one tritium atom in the C-23 to C-29 side chain portion of the sterol. The 3-oxo-bisnorchol-4-en-22-al (XXI) which retained two tritium atoms was converted via the eneamine (XXII) into progesterone (XXIII) with the loss of a further ^{14}C atom (C-22) and a tritium atom. Since it has previously been established that the C-22 hydrogen of poriferasterol is derived from the 2-pro *S* hydrogen of mevalonic acid[24] the above result demonstrated that a tritium atom was located at C-20 in the poriferasterol (XIX). The loss of 80% of the remaining tritium in the progesterone (XXIII) by base catalysed enolization indicated that the remaining tritium atom was located at C-17.



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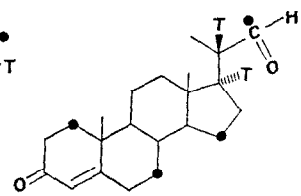
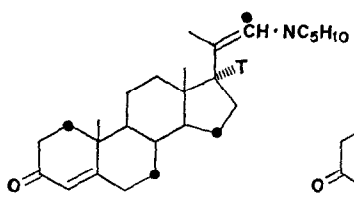
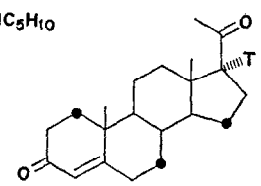


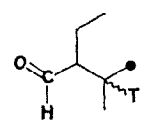
(XXI)



(XXII)



(XXIII)



(XXIV)

The presence of tritium atoms at C-17 and C-20 in poriferasterol (XIX) is in agreement with the results of other workers[16,17] who have demonstrated the same labelling pattern in cholesterol (XVII) biosynthesized by a rat liver preparation from [2-^{14}C, (4*R*)-4-$^3H_1$]-mevalonic acid (XIII). Since cycloartenol (XVIII), the poriferasterol (XIX) precursor, must also be labelled with tritium at C-17 and C-20 these results show that squalene-2,3-oxide (XIV) cyclisation via (XV) to give cycloartenol (XVIII) is accompanied by the expected hydrogen migrations[20] from C-17 to C-20 and C-13 to C-17 and further confirms that the cyclization proceeds by the proposed modification[20] of the squalene-2,3-oxide (XIV) to lanosterol (XVI) cyclization mechanism.[17,25,26]

[22] L. J. GOAD, B. L. WILLIAMS and T. W. GOODWIN, *Europ. J. Biochem.* **3,** 232 (1967).
[23] A. C. SWINDELL and L. J. GAYLOR, *J. Biol. Chem.* **243,** 5546 (1968).
[24] A. R. H. SMITH, L. J. GOAD and T. W. GOODWIN, *Chem. Commun.* 926 (1968).
[25] L. RUZICKA, *Proc. Chem. Soc. Lond.* 341 (1959).
[26] M. JAYME, P. C. SCHAEFER and J. H. RICHARDS, *J. Am. Chem. Soc.* **92,** 2059 (1970).

To determine the position of the third tritium atom in the poriferasterol (XIX) a sample of the labelled compound was treated with ozone to give the C-23 to C-29 portion of the side chain as 2-ethyl-3-methylbutanal (XXIV). One half of the aldehyde (XXIV) was immediately converted into the dimedone derivative which had a $^3H:^{14}C$ atomic ratio showing the presence of one tritium atom (Table 1). The other portion of the labelled 2-ethyl-3-methylbutanal (XXIV) was enolized by treatment with ethanolic KOH before preparation of the dimedone compound which was again found to contain one tritium atom (Table 1). It has previously been established[6] during a study on ergosterol biosynthesis that enolization of 2,3-dimethylbutanal under these basic conditions results in exchange of the C-2 hydrogen atom. Exchange of the C-2 hydrogen atom of 2-ethyl-3-methylbutanal should occur with equal facility and it can therefore be concluded that the present results showed the absence of tritium at this position. Consequently, since C-2 of the aldehyde (XXIV) corresponded to C-24 of the parent sterol there could be no tritium at this position in the poriferasterol (XIX). Although the actual location of the tritium atom in the aldehyde (XXIV) was not determined, it is unlikely mechanistically for it to be elsewhere than at C-3 which arose from C-25 of the poriferasterol (XIX). The presence of the tritium atom at C-25 is therefore consistent with the migration of the C-24 tritium atom of cycloartenol (XVIII) to this position during the side chain alkylation sequence. This observation eliminates from poriferasterol biosynthesis in *O. malhamensis* either a Δ^{24} intermediate (IX route *D*) which would result in tritium elimination or a Δ^{25} compound (XI, route *E*) which would leave the tritium atom at C-24. Both routes *B* and *C* in *O. malhamensis* have been eliminated previously[9] by the use of CD_3-methionine which clearly indicated the involvement of a 24-ethylidene sterol (V). The conversion of several 24-ethylidene sterols into poriferasterol (XIX) by *O. malhamensis*[5,27] and the present results demonstrating the hydrogen migration from C-24 to C-25 provide compelling evidence for the operation of route *A* in this alga. It must be noted, however, that results at present available have not conclusively established the operation of route *A* in other plant species. Investigations are now in progress to check if route *A* does play an important role in other algal and higher plant species or if the alternative mechanisms are more widespread in nature.

EXPERIMENTAL

General procedures. M.ps were determined on a hot stage apparatus and are uncorrected. MS were obtained on an AEI MS12 instrument. IR spectra were determined in KBr discs. TLC was performed using silica gel G or $AgNO_3$ impregnated silica gel as described previously.[5,20] [2-^{14}C]-Mevalonic acid (5·85 mCi/mmol) and (4*R*)-[4-$^3H_1$]-mevalonic acid (116 mCi/mmol) were bought from the Radiochemical Centre, Amersham, England and mixed in the proportions of *ca.* 1:10 on the basis of radioactive content to give the [2-^{14}C, (4*R*)-4-$^3H_1$]-mevalonic acid used for the incubations with *O. malhamensis*. Radioactivity measurements were made on a Beckmann LS200 instrument. Samples were dissolved in 10 ml of scintillation solution containing 2,5-diphenyloxazole (0·5%, w/v) and 1,4-bis-2(4-methyl-5-phenyloxazolyl)benzene (0·03%, w/v) dissolved in toluene. *O. malhamensis* (933/1A) was obtained from the culture collection of Algae and Protozoa, Cambridge and grown in 500 ml conical flasks containing 100–200 ml of the growth medium of Aaronson and Baker.[28] [2-^{14}C, (4*R*)-4-$^3H_1$]-Mevalonic acid was converted into the K salt by dissolving it in dilute K_2CO_3 and sterilized by filtration upon addition to the growth medium. Cultures were grown with constant shaking at 25–27° under illumination and at the end of the incubation period (usually 5–6 days) the cells were harvested by centrifugation. The non-saponifiable lipid was extracted and the squalene, 4,4-dimethyl sterol and poriferasterol fractions obtained as described previously.[5,20]

Incorporation of [2-^{14}C, (4*R*)-4-$^3H_1$]*-mevalonic acid into cycloartenol and* 24*-methylenecycloartanol. O. malhamensis* was cultured in the presence of [2-^{14}C, (4*R*)-4-$^3H_1$]-mevalonic acid (5 μCi of ^{14}C) and the squalene ($^3H:^{14}C$ ratio 11·6, $^3H:^{14}C$ atomic ratio 1:1), 4,4-dimethyl sterol (35 000 dpm of ^{14}C, ratio 11·6)

[27] F. F. Knapp, J. B. Greig, L. J. Goad and T. W. Goodwin, *Chem. Commun.* 707 (1971).
[28] S. Aaronson and H. Baker, *J. Protozool.* **6**, 282 (1959).

and poriferasterol (520 000 dpm of ^{14}C, $^{3}H:^{14}C$ ratio 7·33, $^{3}H:^{14}C$ atomic ratio 3·11:5) fractions isolated. The 4,4-dimethyl sterol fraction was mixed with a mixture of carrier cycloartenol and 24-methylenecycloartanol (50 mg), acetylated (Ac_2O–pyridine) and the acetates separated by TLC on $AgNO_3$–silica gel. The cycloartenyl acetate (520 dpm of ^{14}C, $^{3}H:^{14}C$ ratio 11·8, $^{3}H:^{14}C$ atomic ratio 6·12:6) was crystallized four times to constant specific radioactivity (^{3}H specific radioactivities 290, 310, 300, 310 dpm/mg, final $^{3}H:^{14}C$ ratio 11·4, $^{3}H:^{14}C$ atomic ratio 5·89:6). The 24-methylenecycloartanyl acetate (850 dpm of ^{14}C, $^{3}H:^{14}C$ ratio 11·4, $^{3}H:^{14}C$ atomic ratio 5·89:6) was crystallized three times (^{3}H specific radioactivities 870, 830, 840 dpm/mg, final $^{3}H:^{14}C$ ratio 11·7, $^{3}H:^{14}C$ atomic ratio (6·05:6).

Localization of tritium atoms in poriferasterol (XIX) *biosynthesized from* [2-^{14}C, (4*R*)-4-$^{3}H_1$]-*mevalonic acid. Poriferasterol* (XIX, 590 000 dpm of ^{14}C, $^{3}H:^{14}C$ ratio 6·23, $^{3}H:^{14}C$ atomic ratio 3·08:5) obtained from a second incubation of *O. malhamensis* with [2-^{14}C, (4*R*)-4-$^{3}H_1$]-mevalonic acid (5 μCi of ^{14}C) was degraded to progesterone (XXIII) by the method of Sheppard *et al.*[29] Squalene is generally very poorly labelled from [2-^{14}C]-mevalonic acid by *O. malhamensis* cultures. In this incubation insufficient labelled squalene was obtained to use for the conversion of $^{3}H:^{14}C$ ratios into $^{3}H:^{14}C$ atomic ratios. Therefore, a sample of squalene ($^{3}H:^{14}C$ ratio 10·1) isolated from *Solanum tuberosum* leaves[20] after incubation with a sample of the same batch of [2-^{14}C, (4*R*)-4-$^{3}H_1$]-mevalonic acid was used for this purpose. The $^{3}H:^{14}C$ ratios in this experiment differ slightly from those given in the first experiment since difference batches of [2-^{14}C, (4*R*)-4-$^{3}H_1$]-mevalonic acid were used. *Carrier stigmasterol* (the C-24 epimer of poriferasterol) was added to the labelled poriferasterol (XIX) to give a final wt of 993 mg. and dissolved in dry toluene (17·0 ml). Cyclohexanone (3·0 ml) and aluminium isopropoxide (275 mg) were added and the mixture refluxed for 30 min. The cooled solution was poured into 2 M HCl (50 ml) and extracted with ether. The combined extracts were washed successively with H_2O, 10% (w/v) Na_2CO_3, and H_2O and then subjected to steam distillation. The residue was extracted with ether and the 24-ethylcholesta-4,22-dien-3-one (XX) obtained by chromatography on alumina (anionotropic), Brockmann grade III, developed with increasing percentages of Et_2O in light petroleum. The purified XX (854 mg) had peaks at 1675 and 1620 cm^{-1} in the IR spectrum (584 dpm of ^{14}C/mg, $^{3}H:^{14}C$ ratio 6,20, $^{3}H:^{14}C$ atomic ratio 3·06:5). *The 24-ethylcholesta-4,22-dien-3-one* (XX, 825 mg) was dissolved in CH_2Cl_2 (15 ml) containing pyridine (0·16 ml), cooled to −70°, and treated with O_3 (1·5 eq). HOAc (2 ml) and Zn dust (250 mg) were added, the temp. raised to 10° and the mixture stirred for 90 min. The recovered reaction products were subjected to chromatography on alumina, Brockmann, grade III eluted with Et_2O–light petroleum mixtures to give 3-oxobisnorchol-4-en-22-al (XXI). This material was pure as judged by silica gel TLC and was crystallized from EtOH, m.p. 154–156° (lit.,[29] 156°). The IR spectrum of (XXI) included bands at 2870, 2750, 1730, 1670 and 1620 cm^{-1} (605 dpm of ^{14}C/mg, $^{3}H:^{14}C$ ratio 5·30, $^{3}H:^{14}C$ atomic ratio 2·1:4). *3-Oxobisnorchol-4-en-22-al* (XXI, 234 mg) was dissolved in benzene (5 ml) and piperidine (0·075 ml) and anhydrous K_2CO_3 (100 mg) added. After refluxing for 3 hr the solution was filtered, the residue washed with benzene and the combined washings and filtrate evaporated to dryness to give the crude enamine (XXII). This was separated by preparative TLC on silica gel ($CHCl_3$) to give the unstable 22-(*N*-piperidyl)-bisnorchola-4,20(22)-dien-3-one (XXII) which was crystallized from MeOH by cooling to −20° (m.p. 135–136°, lit.,[30] 136°, 463 dpm of ^{14}C/mg, $^{3}H:^{14}C$ ratio 3·65, $^{3}H:^{14}C$ atomic ratio 1·4:4). *Sodium dichromate dihydrate* (250 mg) was dissolved in HOAc (1·25 ml), benzene (0·9 ml) added, and the mixture stirred and cooled in an ice bath. A solution of the crude enamine (XXII, 163 mg) in benzene (1·25 ml) was added over a period of 1 hr and the stirring continued for a further 2 hr after which MeOH (0·5 ml) was added. After conventional work up, crude progesterone (XXIII) was recovered and TLC on silica gel gave pure progesterone (XXIII, $^{3}H:^{14}C$ ratio 3·68, $^{3}H:^{14}C$ atomic ratio 1·09:3). The MS had peaks at *m/e* 314 (M^+), 299, 296, 272, 244, 229 and 124 in agreement with the literature[31] and a reference sample. Carrier progesterone (40 mg) was added and the mixture crystallized four times which left the $^{3}H:^{14}C$ ratio virtually unchanged at 3·64. The crystallized progesterone (XXIII) was dissolved in 8% (w/v) methanolic KOH (5 ml) and 50% (v/v) aq. MeOH (5 ml) added. After 4 hr reflux in N_2 the MeOH was removed in N_2 and the aqueous mixture extracted with Et_2O. The Et_2O extract was washed with H_2O to neutrality, dried and evaporated to dryness to give an oil which was counted without further purification[16] ($^{3}H:^{14}C$ ratio 0·75, $^{3}H:^{14}C$ atomic ratio 0·22:3). Another sample of *poriferasterol* (XIX, 2×10^5 dpm of ^{14}C) was mixed with carrier poriferasterol (237 mg), dissolved in warm HOAc (2 ml), cooled to give a fine suspension and then treated with O_3. After addition of Zn dust (500 mg) the mixture was stirred for 90 min at 10°, filtered and the residue washed with HOAc (3 ml). The bulked filtrates were added to H_2O (70 ml) and the mixture steam-distilled. The 2-ethyl-3-methylbutanal (XXIV), produced by the reductive ozonolysis of the Δ^{22} bond of poriferasterol (XIX), distilled over as oily drops with the H_2O. At completion of the distillation the distillate was carefully neutralized with aq. NaOH and divided into two portions. To one portion was added dimedone (200 mg) in ethanol (2 ml) to render the final solution 25% (v/v) with respect to EtOH. The dimedone derivative which slowly precipitated was purified by TLC on silica gel developed with $CHCl_3$ and

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crystallized from aq. EtOH (m.p. 129°, $^{3}H:^{14}C$ ratio 11·4, $^{3}H:^{14}C$ atomic ratio 1·12:1). The second portion of the aldehyde (XXIV) solution was added to 3 vol. of absolute EtOH and NaOH added to give a concn of 10% (w/v)[6] and the solution allowed to equilibrate for 24 hr at 20°. After careful neutralization with HOAc the dimedone derivative was obtained as above ($^{3}H:^{14}C$ ratio 10·1, $^{3}H:^{14}C$ atomic ratio 1·00:1).

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