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Biosynthesis of Ergosta-4,6,8(14),22-tetraen-3-one. A Novel Oxygenative Pathway[1]

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Specifically (tritium) labeled precursors (VIII, X, XIV, XV, and XVI), upon feeding to *Penicillium rubrum*, are incorporated into ergosta-4,6,8(14),22-tetraen-3-one (IV) to the extent of 14.2, 4.5, 11.4, 16.3, and 5.5% respectively. Proof that the ergostane skeleton was incorporated intact was afforded by a chemical-biosynthetic cycle, the latter stages of which entailed reduction of isolated (IV) to ergosterone (VIII), followed by removal of the label through base-catalyzed exchange. A search of the growth medium of *P. rubrum* revealed the presence of nonartefactual ergosterol epidioxide (XIII) and ergosta-6,22-dien-3β,5α,8α-triol (XVIII). The incorporation data are consistent with a set of multiple pathways with no unique biosynthetic sequence apparent.

The utilization of molecular oxygen in secondary biosynthesis takes several forms, of which those pathways dependent upon incorporation of the intact oxygen molecule constitute one important variant (*1*). Since the discovery by Hayaishi (*2*) of the oxidative cleavage of catechol to form *cis, cis*-muconic acid, a number of biological oxidations have been brought to light which depend upon mediation by a dioxygenase (*3*). The proposal that a 1,2-dioxetane intermediate can participate in these processes is supported by labelling experiments with $^{18}O_2$ (*4*), and also by the chemical properties of certain, stable dioxetanes which have recently become available (*5*).

In contrast, the role of 1,4-dioxides in secondary biosynthesis has seemed much less secure, even though these substances, in natural form, actually represent a well-characterized group of metabolites. Thus, ascaridole (I) (*6*), α-phellandrene epidioxide (II) (*7*), and ergosterol epidioxide (XIII) (*8*) are, so far as can be discerned from present criteria, authentic products of secondary metabolism,[5] yet there is uncertainty as to what end, if any, this biological functionalization is put. A suggestion that the epidioxide (III) of 7-dehydrocholesterol is an intermediate in the nonphotochemical biosynthesis of vitamin D_3 in Atlantic striped bass (*10*) has not been fully substantiated

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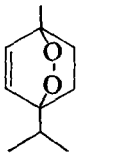
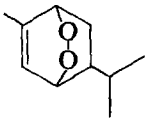
[5] It has been shown that, in the absence of careful controls, artefactual ergosterol epidioxide can be mistaken for the natural product (*9*).

(*11*). A claim by Topham and Gaylor (*12*) that ergosterol epidioxide (XIII) is converted by a yeast enzyme to ergosterol (VI) is to be contrasted with an earlier report which failed to detect enzyme-catalyzed conversion of (III) to cholesterol (*13*). We here present the results of *in vivo* experiments with *Penicillium rubrum*, in which certain precursors, including ergosterol epidioxide, were tested for incorporation into ergosta-4,6,8(14),22-tetraen-3-one (IV) (*14*).

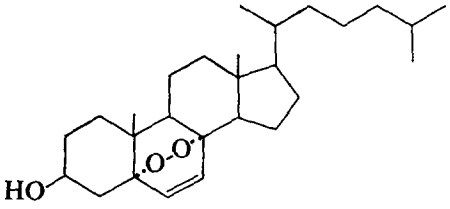
In the course of isolation studies with *P. rubrum*, it became apparent that, in addition to rubratoxin-A and -B (*15*) and certain pigments (*16*), the organism was producing appreciable quantities of a nonpolar substance which could be readily recognized in a chromatographic medium by its intense, blue fluorescence under uv illumination.



(I)



(II)



(III)

Isolation of this material allowed its identification as (IV), previously obtained by synthesis (*17*).[6] Subsequently, (IV) has been found to occur in the yeast *Candida utilis* (*18*), in the fungus *Fomes officinalis* (*19*), and in the bioluminescent mushroom *Lampteromyces japonicus* (*20*). Of particular interest is the finding that fluorescence emission of (IV) at 528 nm is coincident with the wavelength of bioluminescence of *L. japonicus*.

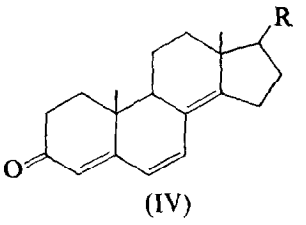
In considering the biogenesis of (IV), it seemed plausible that this metabolite could be formed via an oxygenative pathway followed by dehydration; a possible intermediate in this process might then be ergosterol epidioxide (XIII) since this compound is at the oxidation level of (IV). Moreover, several economical pathways can be envisaged whereby (XIII) could be transformed to (IV). In order to test this hypothesis, the synthesis of tritium-labeled precursors and their incubation with *P. rubrum* cultures was undertaken.

SYNTHESIS OF PRECURSORS

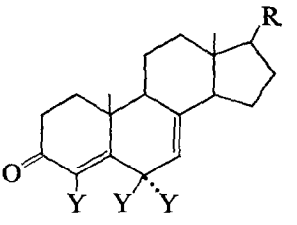
Ergosterone (V), prepared by the Oppenauer oxidation of ergosterol (VI) (*21*), provided a convenient vehicle for introduction of isotopic hydrogen into the set of

[6] We are indebted to Professor D. H. R. Barton for providing a sample of (IV) and its 2,4-dinitrophenylhydrazone.

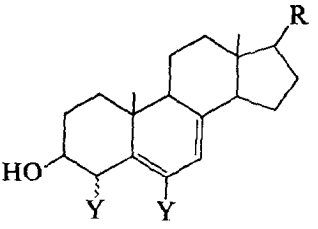
precursors required for feeding experiments with *P. rubrum*. Formation of the enolate of (V) with sodium methoxide in tetrahydrofuran, followed by a quench with D_2O, afforded a deuterated species which was predominantly $[^2H]_3$-$\Delta^{4,7}$-ergosterone (VII) as judged from mass spectral data (M^+ at *m/e* 397). Disappearance of the one-proton



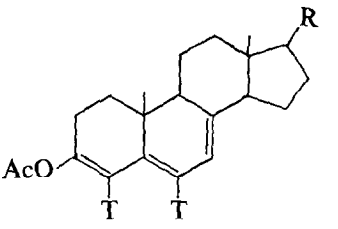
(IV)



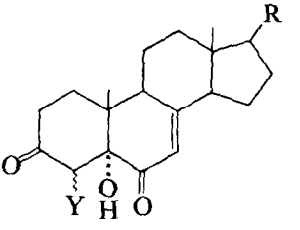
(V), Y = H
(VII), Y = D
(VIII), Y = T



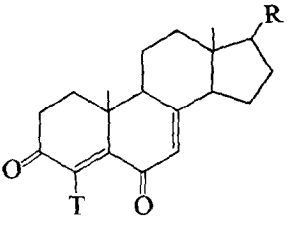
(VI), Y = H
(X), Y = T



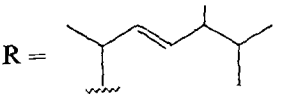
(IX)



(XI), Y = T
(XXV), Y = H



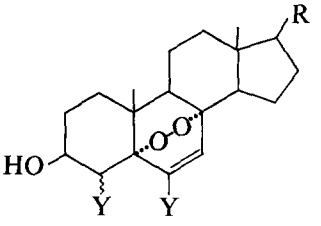
(XII)



singlet originally present at δ5.77 confirmed the incorporation of deuterium at C-4 and the absence of the AB quartet centered at δ 2.9, corresponding to protons at C-6, indicated that deuterium substitution at this site had occurred from both the α and β sides of the enolate. Tritium was introduced into (V) in an analogous fashion using HTO, with the label in this case distributed between C-4, C-6α, and C-6β sites (*vide infra*).

Labeled ergosterone (VIII) ($7.02.10^7$ dpm/mmole) was converted to enol acetate (IX) ($6.55.10^7$ dpm/mmole) with acetic anhydride in pyridine (*22*), the discriminative isotope effect in this reaction assuring selective retention of the label at C-6. Reduction of (IX) with sodium borohydride in aqueous dioxane gave [4,6-^{3}H]-ergosterol (X) (*23*) ($5.18.10^7$ dpm/mmole) in an overall mass yield of 38% from unlabeled ergosterol and an overall radiochemical yield of 74%. Because of its possible importance in the interpretation of incorporation results, it was necessary to determine the relative tritium distribution between C-4 and C-6 in (X). This was accomplished by oxidation of (X) with chromium trioxide in acetic acid to hydroxydiketone (XI) (*24*), which was found to have lost 62% of the tritium originally present. The residual label at C-4 was shown to be equally distributed between α and β configurations by dehydration of (XI) to (XII) (*25*), a process in which *trans*diaxial elimination leads to a product having lost a further 19% of the label.

A noteworthy feature of the sequence leading from (VI) to (X) is the lack of regioselectivity and stereoselectivity in the incorporation of tritium into (V). This is to be contrasted with the exchange reaction of testosterone where the incorporation of label is notably specific in regard to site and configuration (*26*). The more highly conjugated enolate derived from (V) presumably allows greater opportunity for thermodynamic control to effect equilibration of label among sites. The same enolate must be produced in the borohydride reduction of (IX) since saponification of the enol acetate constitutes the first stage in this reaction (*27*); here, however, the equal distribution of label between 4α and 4β sites must reflect kinetic control since there is negligible tritium loss in the



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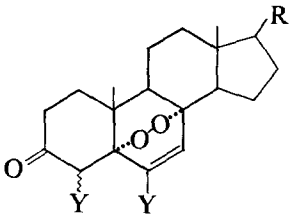
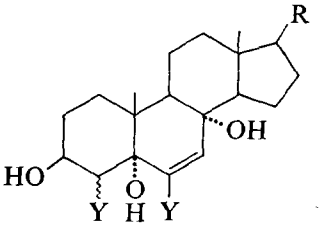
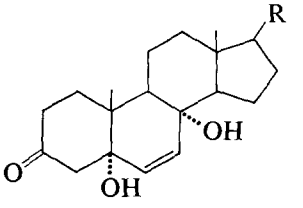
(XIV), Y = T



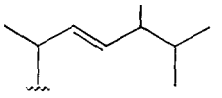
(XVI), Y = T
(XIX), Y = H



(XV), Y = T
(XVIII), Y = H



(XVII)

R = 

overall reduction step. Thus, in contrast to the usual preference for replacement of isotopic hydrogen from the axial direction (*28*), the flattened structure of the enolate of (V) permits proton addition at C-4 from either side. Because borohydride reduction proceeds much faster than double-bond migration (*27*), the major product from (IX) is the $\Delta^{5,7}$-dienol; for this reason preparation of (X) via the enol acetate (IX) is much preferable to the direct reduction of (VIII).

Tritiated ergosterol (X), obtained by dilution with cold material (ca. five times) of the reduction product from (IX), provided the starting material for the synthesis of other labeled precursors. Thus, photochemical oxygenation of (X) using methylene blue as sensitizer afforded epidioxide (XIV) (*29*) ($1.46.10^7$ dpm/mmole), which was reduced with zinc in ethanolic potassium hydroxide to triol (XV) (*30*) ($3.44.10^6$ dpm/mmole). Oxidation of (XIV) with Jones' reagent gave the 3-ketoepidioxide (XVI) (*31*) ($1.18.10^7$ dpm/mmole). Attempts to prepare ketodiol (XVII) by oxidation of (XVIII) were unsuccessful; Jones' reagent led principally to the hydroxydiketone (XXV), previously obtained from the oxidation of ergosterol (VI), whereas oxidation with chromium trioxide in pyridine effected concomitant dehydration to give (IV). In fact, this latter method has proved to be the most efficient means available for the synthesis of (IV) and was used for the preparation of material for dilution of radiolabeled tetraenone. Oxidation of (XVIII) with dicyclohexylcarbodiimide in dimethyl sulfoxide (*32*) gave (IV), though in lower yield.

INCORPORATION OF PRECURSORS

Penicillium rubrum was grown in submerged culture on the standard liquid medium previously described (*33*). Innoculation was from colonies grown on agar or by direct introduction of a suspension of spores into the medium. The former method was found to be generally superior in giving a more homogeneous culture with greater efficiency in the assimilation of precursors. The optimum period for administering precursors was found to be 4–5 days after innoculation. Tetraenone (IV) appeared at 10–12 days of growth and reached a maximum concentration in the medium (ca. 50 mg/liter) after 18–21 days. At this point, cellular material was ruptured and the solids were separated by centrifugation. Tetraenone (IV) was isolated by extraction into chloroform followed by column chromatography. After purity had been rigorously established by tlc, (IV) was quantitatively assayed by means of its uv absorption band at 348 nm (ε 26 500). Tritium content was then determined by scintillation counting.

As a further check on the accuracy of the radioassay and also to ascertain that incorporation of precursors was taking place without significant scrambling of label, a large-scale feeding with [4,6-^{3}H]-ergosterol (X) was carried out with the objective of obtaining sufficient tetraenone for a degradative study. The isolated tetraenone (IV) was crystallized to constant specific activity and its activity verified against that of chromatographically isolated (IV). The tetraenone, after dilution with cold material, was reduced with lithium–ammonia to [4,6-^{3}H]-ergosterone (VIII), which underwent exchange with water in the presence of base resulting in complete removal of all of the label. The overall sequence therefore completes a chemical-biosynthetic cycle which establishes, at least for the ergosterol feeding, that incorporation took place without

metabolic degradation. It seems likely that the same holds true for precursors (XIV, XV, and XVI) but this has not been proven rigorously.

RESULTS AND DISCUSSION

The results of feeding experiments with (VIII, X, XIV, XV, and XVI) are presented in Table 1. All five compounds are incorporated into (IV) at levels which assure them

TABLE 1

INCORPORATION OF PRECURSORS INTO ERGOSTA-4,6,8(14),22-TETRAEN-3-ONE IN *Penicillium rubrum*

Precursor	Spec act of precursor, dpm/mmole	Spec act of (IV)[a] dpm/mmole	Incorporation[b] %
(VIII)	4.67×10^8	6.62×10^7	14.2
(X)	1.17×10^7	4.43×10^5	4.5
(XIV)	1.35×10^7	1.30×10^6	11.4
(XV)	3.39×10^6	4.64×10^5	16.3
(XVI)	1.04×10^7	4.84×10^5	5.5

[a] Average of duplicate runs.

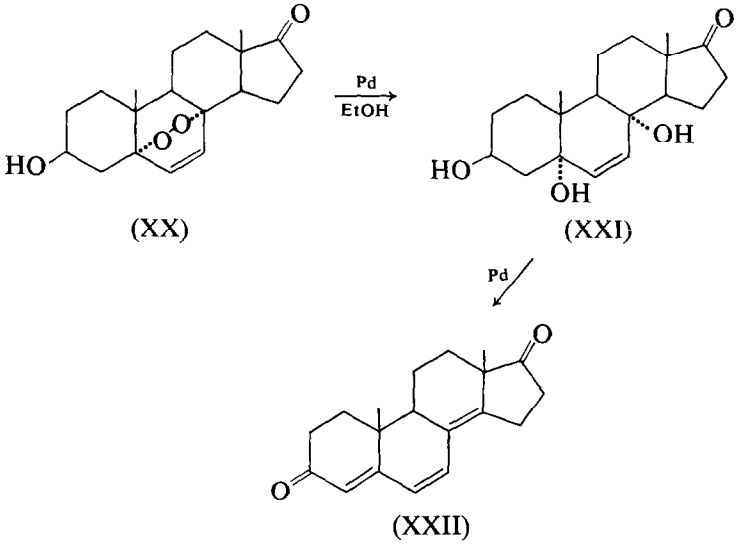
[b] Includes a correction for loss of 19% radioactivity from C-4 in formation of (IV).

of a role as precursor, although ergosterone (VIII), ergosterol epidioxide (XIV), and triol (XV) are somewhat better precursors of (IV) than either the ketoepidioxide (XVI) or ergosterol itself. The incorporation of (XIV) provides firm proof that a 1,4-dioxide can be metabolized in a biosynthetic process, a finding that is supported by a considerable body of circumstantial evidence (*34*). However, incorporation of ketoepidioxide (XVI) and of ergosterone (VIII) indicates that several pathways must be operative in producing (IV). The difficulties inherent in establishing specific pathways in secondary biosynthesis have been well demonstrated in the attempts to delineate the biosynthesis of ergosterol (*35*), where the nonspecificity of enzymes and a latitude in the sequencing of reactions have led to the conclusion that there can be no unique route to this metabolite. In fact, our results can be well accommodated by the type of metabolic grid of intersecting pathways discussed by Bu'Lock (*36*).

Concurrently with precursor–incorporation studies, a search of the *P. rubrum* growth medium was conducted for intermediates along the proposed biogenetic pathway. Epidioxide (XIII) was found (ca. 50 mg/liter) at 10 days of growth although none (<0.01 mg/liter) was present at the time (19 days) when tetraenone was isolated. A blank run containing all of the constituents of the growth medium and to which ergosterol was added failed to produce a trace of (XIII) when not inoculated. Cultures were grown in darkness and harvested under subdued light; hence it is most unlikely that (XIII) is produced by an artefactual, photochemical oxidation in the present case.

A careful survey of the filtrate also revealed the presence of a small amount of triol (XVIII), but neither ketoepidioxide (XIX) nor ergosterone (V) could be detected. A somewhat unexpected result emerged when relatively large doses (>500 mg in 250 ml cultures) of (XIII) were administered; synthesis of (IV) was terminated and a closely related, as yet unidentified, metabolite was produced instead. Apparently, (XIII) is able to effect control over a more advanced step in the biosynthetic sequence and is diverted from the pathway leading to (IV). The incorporation of (X) into (XIII), and (XIV) into (XVIII), as well as other possible unitary changes, are under study and will be reported subsequently.

In vivo conversion of (XIII) to (XVIII) and thence (IV) has a remarkably close chemical parallel in a sequence recently reported by Johns (*37*), in which it was shown (Scheme I) that treatment of (XX) with palladium in ethanol resulted in a 70% conversion to triol (XXI), whereas more prolonged exposure (10 days) gave up to 60% of trienone (XXII)[7].

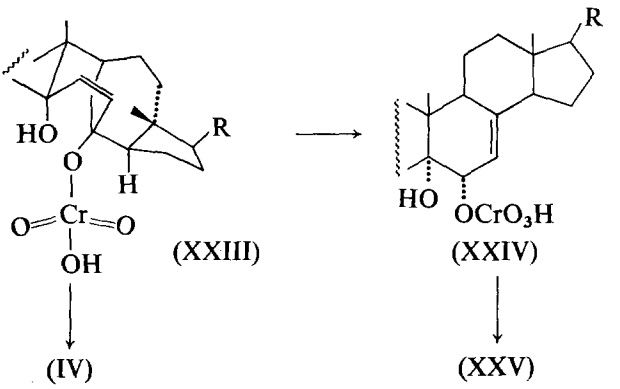


(XX) (XXI) (XXII)

Scheme I

The facile loss of water accompanying the dehydrogenation of (XXI) takes place despite the fact that generation of the 8,14-double bond requires a formal *cis* elimination. The ease with which dehydration occurs at this site in (XXI) and in (XVII) can be traced to steric repulsions arising from the enforced boat conformation of the B ring. The interactions which are particularly severe are those between (i) "bowsprit-flagpole" hydroxyl groups at C-5 and C-8, (ii) the methyl group at C-10 and the C-12 methylene group, and (iii) the methyl group at C-13 and the C-7 hydrogen. Conversion of C-8 to sp^2 configuration provides substantial relief from each of these congestions and, for (XVIII), is shown (Scheme II) as proceeding via 1,2-elimination of chromate ester (XXIII) to give (IV) and via allylic rearrangement of (XXIII) to produce (XXIV), from which hydroxydiketone (XXV) is derived.

[7] The analogous sequence with ergosterol epidioxide has been reported in outline (*38*).



Scheme II

The incorporation of (X) and (XIV) into (IV) suggests that *P. rubrum* must possess a dioxygenase capable of effecting addition of an intact oxygen molecule to the diene system of ergosterol.[8] This is of particular significance in view of the claim (*40*), recently brought into question (*41*), that soybean lipoxidase accomplishes oxygenation of tetracyclone to its epidioxide. As a possible chemical model for this oxygenation, ergosterol was allowed to react with the oxygen complex of tristriphenylphosphinechlororhodium (*42*). Although the reaction was slow and no epidioxide could be detected, virtually the sole product was the tetraenone (IV). A trace of (IV) was observed in the presence of the nonoxygenated rhodium complex, suggesting that the reaction proceeds, in part, through a dehydrogenative pathway. A more detailed evaluation of this reaction and of the biological oxygenation must await an extended study.

EXPERIMENTAL

Melting points were measured on a Kofler hot-stage microscope and are uncorrected. Ultraviolet spectra were measured on a Cary Model 14 recording spectrophotometer with methanol as the solvent. Radioisotope concentrations were taken from a printed readout from a Packard Model 3003 liquid scintillation spectrometer. The compound to be assayed was dissolved in a scintillation cocktail derived from 15.1 g of 2,5-diphenyloxazole (PPO Scintanalyzed from Fisher Scientific Co.) in 1 liter of toluene and counting was carried out simultaneously over three channels by repeating at 1-min intervals during 3 hr. Radiopurity was established by crystallization to constant activity. The identity of labeled compounds was established by mixed mp and infrared spectral comparison with cold material.

Incubation Procedure

Colonies of *P. rubrum* (strain P-12)[9] were first grown on a sterilized agar medium consisting of glucose (25 g/liter), yeast extract (Difco, 6.4 g/liter), KH_2PO_4 (10.0

[8] The possible intervention of singlet ($^1\Delta_g$) oxygen in processes of this type has been recognized (*39*).

[9] We are indebted to Professor D. Wang, Department of Biology, Massachusetts Institute of Technology, for providing a soil culture of this organism.

g/liter), $MgSO_4$ (1.0 g/liter), and a standard trace element solution (*43*). The liquid growth medium (450 ml), contained in 2.8 liter Fernbach flasks, was innoculated after sterilization by transfer of 5-day-old colonies of *P. rubrum* dispersed in a small amount of the growth medium. The cultures were placed on a rotary shaker and incubated at 86° F. Precursors (ca. 50 mg) were dissolved in 5–10 ml of ethanol and were administered at 4–5 days of growth. In the case of (XV), the precursor was administered as a finely ground suspension in ethanol. No pH controls were used so that the pH of the medium, initially ca. 5, dropped to 3.0 during 2 days and thereafter remained approximately constant.

Isolation of Ergosta-4,6,8(14),22-tetraen-3-one (IV)

After 18–20 days of growth, the culture was filtered. The mycelial mat remaining in the funnel was broken up and placed in a blending cup with 50 ml of chloroform. The mixture was stirred rapidly for 5 min and then the chloroform extract was combined with the aqueous filtrate. The mixture was centrifuged at 10 000–12 000 rpm for 10 min. The lower layer was drawn off and dried, and the chloroform was removed *in vacuo* leaving a yellow, oily residue.

The crude extract so obtained was chromatographed on alumina (Woelm Activity II). The tetraenone (IV) could be detected on the column by means of its intense blue fluorescence under uv light and was eluted with a 1:1 benzene–ether mixture. Crystallization from ethanol gave (IV) as pale yellow prisms, mp 112–113° (lit. (*17*) 113–114°); $[\alpha]^{24}D$ + 592 (*c* 0.82,$CHCl_3$) (lit. (*17*) + 588); ir (Nujol) 1670, 1640, 1595 cm^{-1}; uv 348 nm (ε 26 500); nmr (δ, $CDCl_3$) 5.18 (2 H,m), 5.57 (1 H,s), 5.94 (1 H,d, $J = 9$ Hz), 6.44 (1 H,d, $J = 9$); mass spectrum *m/e* 392.305 (parent, calcd for $C_{28}H_{40}O$ 392.308), 268.184 (base peak, calcd for $C_{19}H_{24}O$ 268.183, from cleavage of side chain). Ketone (IV) gave a 2,4-dinitrophenylhydrazone derivative as dark red needles (from ether), mp 226–229° dec (lit. (*17*) 231–234° dec).

Ergosta-4,7,22-trien-3-one-4,6α,6β[^3H] (VIII)

To a solution of 0.50 g (1.25 mmole) of (V) in 15 ml of dry tetrahydrofuran was added 0.10 g (1.85 mmole) of freshly prepared sodium methoxide. The mixture was stirred at 50° for 10 min and, after cooling to room temp., 0.5 ml of HTO (1 C/ml) was added followed by 2 ml of 6 *N* hydrochloric acid. The mixture was poured into ice-water and the precipitated solid was collected by suction filtration. The solid was crystallized from acetone to give 0.43 g (85%) of (VIII), mp 130–133° (lit. (*21*) 128–130°).

A parallel deuteration experiment carried out under similar conditions using D_2O gave (VII); nmr ($CDCl_3$) δ 0.65 (3 H,s), 0.88 (6 H,d, $J = 7$), 0.95 (3 H,d, $J = 7$), 1.07 (3 H,d, $J = 7$), 1.20 (3 H,s), 1.1–2.4 (broad), 5.19 (3 H,m) (a broad 1 H,d ($J = 18$) at 2.62, a broad 1 H,d ($J = 18$) at 3.14, and a 1 H,s at 5.76 in the spectrum of (V) were absent from that of (VII)). The mass spectrum of (VII) showed 88% d_3, 10% d_2, 2% d_1, and ~0% d_0 species.

3-Acetoxy-3,5,7,22-tetraene-4,6[^3H] (IX).

A solution of 7.0 g (17.7 mmole) of (VIII) in 5 ml of dry pyridine containing 5 ml of acetic anhydride was heated under reflux for 3 hr. Upon cooling, a solid separated which was collected by filtration and crystallized from a 1:1 mixture of methanol and

ethyl acetate until a mp 148–151° (lit. (*22*) 146°) was obtained. The yield was 5.95 g (85%).

Ergosterol-4α,4β,6[^3H] (X)

A mixture of 6.0 g (13.6 mmole) of (IX) and 1.9 g of sodium borohydride in 200 ml of freshly distilled dioxane containing 20 ml of water was warmed to 96° during 45 min. The resulting solution was maintained for a further 45 min at this temp. and then poured into water. The precipitated solid was collected by filtration, dried under suction, and crystallized from chloroform–methanol to give 4.52 g (75%) of (X), mp 160–162° (lit. (*23*) 160–162°).

5α-Hydroxyergosta-7,22-dien-3,6-dione-4α,4β [^3H] (XI)

To a stirred solution of 2.45 g (6.19 mmole) of (X) (1.31×10^5 dpm/mmole) in 80 ml of acetic acid at 50° was added a solution of 2.2 g of chromium trioxide in 15 ml of 90% acetic acid. After 5 min the mixture was poured into 200 ml of water and extracted with ether. The ether extract was washed with water and dried ($MgSO_4$). The solvent was removed *in vacuo* and the residue was crystallized from ethanol to give 0.89 g (33%) of (XI), mp 249–252° (lit. (*24*) 249° dec) (0.51×10^5 dpm/mmole).

5α,8α-Epidioxyergosterol-4α,4β,6[^3H] (XIV)

A solution containing 7.02 g (0.018 mole) of (X) and a trace of methylene blue in 400 ml of ethanol was irradiated in a Pyrex vessel with a 275-W GE Sunlamp as a continuous stream of oxygen was passed through the mixture. After 6 hr the mixture was poured into water and extracted with ether. The ether extract was filtered through charcoal and dried ($MgSO_4$). The residue after removal of the solvent was crystallized twice from ethanol to give 3.80 g (50%) of (XIV), mp 174–177° (lit. (*29*) 178°).

Ergosta-4,7-dien-3,6-dione-4[^3H] (XII)

A mixture of 0.45 g (1.10 mmole) of (XI) (0.51×10^5 dpm/mmole) and 50 ml of 10% ethanolic potassium hydroxide was stirred at room temp. for 10 hr. The dark red solution was acidified with 6 *N* hydrochloric acid until the color was discharged to a bright yellow and the mixture was extracted with ether. The ethereal solution was washed with sat. brine and the solvent was removed to leave a yellow gum. This material was purified by preparative tlc on silica (3% methanol in benzene), eluting with chlorform, and was crystallized from ether–hexane to give 0.05 g (12%) of (XII) as yellow needles, mp 144–146° (lit. (*25*) 145–146°) (0.25×10^5 dpm/mmole).

Ergosta-6,22-dien-3β,5α,8α-triol-4α,4β,6[^3H] (XV)

To a solution of 2.50 g (5.84 mmole) of (XIV) in 250 ml of 5% ethanolic potassium hydroxide was added 25 g of granular zinc. The mixture was then heated under reflux for 1 hr and filtered while hot. The filtrate was poured into water and the precipitated solid was collected and dried. Crystallization from ethanol and then from ethyl acetate gave 1.97 g (79%) of (XV), mp 223–225° (lit. (*30*) 227° dec).

5α,8α-Epidioxyergosta-6,22-dien-3-one-4α,4β,6[^3H] (XVI)

To a solution of 0.204 g (0.48 mmole) of (XIV) in 7 ml of acetone at 0° was added dropwise 0.5 ml of Jones' reagent. During the addition the temp. of the mixture was

maintained below 10°. The mixture was stirred at room temp. for 15 min after addition was complete and 0.2 ml of methanol was then added. After stirring for a further 15 min, the mixture was poured into water and extracted with ether. The ether extract was washed with sat. sodium bicarbonate, filtered through charcoal, and dried ($MgSO_4$). The residue after removal of the solvent was crystallized twice from ethanol to give 0.082 g (40%) of (XVI), dec ca. 165° (lit. (*31*) 162–180° dec).

Isolation of Ergosterol Epidioxide (XIII) and Ergosta-6,22-dien-3β,5α,8α-triol (XVIII)

Four 450 ml, 10-day cultures were combined and filtered. The filtrate was extracted with ethyl acetate and the extract was washed thoroughly with sat. sodium bicarbonate. The residue after removal of the solvent at room temp. *in vacuo* was taken up into benzene and chromatographed on alumina (Woelm Activity IV). The epidioxide (XIII) (41 mg) was eluted with ether and was crystallized from ethanol. Comparison of tlc characteristics, mp, and ir and mass spectra with those of synthetic (XIII) (*29*) established identity.

Further elution of the column with ethyl acetate gave material which showed tlc behaviour identical with that of triol (XVIII). Crystallization by slow evaporation of ethyl acetate gave 6 mg of (XVIII), identified by mp and mass spectral comparison with synthetic (XVIII) (*30*).

Ergosta-4,6,8(14),22-tetraen-3-one (IV) from Triol (XVIII)

To a stirred suspension of 0.22 g (0.51 mmole) of (XVIII) in 5 ml of dry pyridine was added 20 ml of Cornforths' reagent (*44*). The mixture was stirred at room temp. for 2 hr during which the triol gradually dissolved. The mixture was poured into water and extracted with ether. The ethereal extract was washed well with 10% hydrochloric acid followed by sat. cupric sulfate solution. The residue after removal of the solvent was taken up into ethanol and the resulting solution was refrigerated for 48 hr. The crystalline solid which had been deposited was filtered and the filtrate was evaporated to dryness. The residue was taken up into benzene and chromatographed on alumina. Elution with benzene–ether (4:1) gave a yellow oil which crystallized upon standing and which was recrystallized from ethanol to give 0.062 g (31%) of (IV), mp 113–114° (lit. (*17*) 113–114°).

Reduction of Ergosta-4,6,8(14),22-tetraen-3-one-4,6[^3H] to Ergosta-4,7,22-trien-3-one-4,6α,6β[^3H] (VIII)

To a solution of 15 mg of lithium in 50 ml of dry ammonia was added slowly a solution of 80 mg (0.20 mmole) of (IV) [2.58×10^5 dpm/mmole, isolated from a feeding with (X)] in 18 ml of *t*-butanol and 10 ml of ether. The reaction mixture turned from dark blue to yellow at the end of the addition. After stirring for 30 min, a sat. solution of ammonium chloride was added and the ammonia was allowed to evaporate. The mixture was extracted with ether and (VIII) was isolated by preparative tlc on silica in 1% methanol–benzene. Crystallization from acetone gave 18 mg (23%) of (VIII) (6.31×10^4 dpm/mmole).

To a solution of 18 mg (0.046 mmole) of (VIII) in 5 ml of ether was added a solution of 10 mg of sodium methoxide in 15 ml of methanol. The mixture was shaken thoroughly and then acidified with 10% hydrochloric acid. The ether layer was washed with water

and the exchange procedure was then repeated twice more. Ergosterone (V) was isolated by preparative tlc on silica in 2% methanol–benzene and was admixed with 50 mg of cold (V). The cocrystallized ergosterone (from acetone) was found to possess radioactivity not significantly greater than background.

Ergosta-4,6,8(14),22-tetraen-3-one (IV) from Ergosterol[10]

A stream of oxygen was passed for 1 hr into a mixture containing 1.00 g (2.52 mmole) of ergosterol and 25 mg of tristriphenylphosphinechlororhodium in 20 ml of dry diglyme. The mixture was allowed to stand overnight and was then diluted with water. The mixture was extracted with ethyl acetate and (IV) was isolated by preparative tlc on silica as described above. The yield of (IV) was 36 mg (4%) and identity was established by comparison of ir and mass spectra with those of authentic material.

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