Biosynthesis of Ergosta-6(7),8(14),22(23)-trien-3β-ol by *Gibberella fujikuroi:* Its Importance to Ergosterol's Metabolic Pathway

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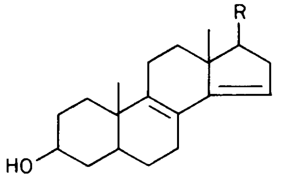
A series of ergostatrienes: ergosta-8(9),14(15),22(23)-trien-3β-ol (**1**), ergosta-5(6),8(9),22(23)-trien-3β-ol (**2**), ergosta-5(6),7(8),22(23)-trien-3β-ol (**3**), and ergosta-6(7),8(14),22(23)-trien-3β-ol (**4**), have been isolated and characterized by chromatographic and spectral methods from mycelia of *Gibberella fujikuroi* and through appropriate feeding and trapping experiments the sequence (**1**)→(**2**)→(**3**) has been demonstrated with (**4**) being an unmetabolized end product of biosynthesis.

Ergosterol biosynthesis in algae and fungi is known to proceed by multiple routes, however, none of the biosynthetic schemes show (**4**) as an intermediate.[1-6] Mechanistic considerations based on feeding and trapping experiments using animal and fungal systems coupled with the lack of having found to date, a naturally occurring $\Delta^{6(7)}$-sterol[7] seemingly relegated (**4**) to the status of a demonstrable chemical by-product following acid-catalysed formation of (**1**) from (**3**).[8] In the course of delineating the reaction sequences and function of 24β-methylsterols produced by *Gibberella fujikuroi*, we observed that this fungus synthesized during logarithmic growth three non-interconvertible biosynthetic end products: 24β-methylcholesta-5,22-diene-3β-ol (brassicasterol), 24β-methylcholesterol, and ergosterol (free sterols which predominate in mycelial membranes).[9,10] When the mycelia harvested in the early stationary phase of growth were examined by g.l.c.–mass spectrometry (m.s.), three additional ergostatrienes from the saponified steryl ester (SE) fraction (M^+ 396) were observed in trace amounts in the two principal g.l.c. peaks which we had assigned originally to (esterified) brassicasterol and ergosterol.[10] These sterols were monitored by reversed-phase h.p.l.c. equipped with a multiple wavelength diode-array



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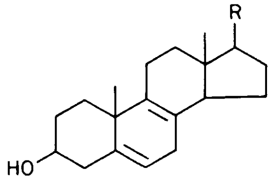

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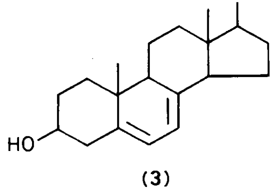

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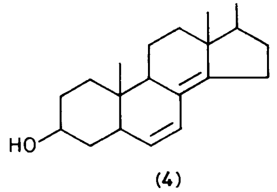

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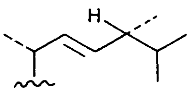

Table 1. Chromatographic and spectral data for four ergostatrienes isolated from *G. fujikuroi.*

Analysis		Values for sterol			
		(1)	(2)	(3)	(4)
^{1}H N.m.r.[a]	18-H	0.83 (br. s)	0.67 (s)	0.63 (s)	0.64 (s)
	19-H	0.99 (s)	1.19 (s)	0.95 (s)	0.90 (s)
	21-H	1.03 (d)	1.03 (d)	1.04 (d)	1.03 (d)
	26-H	0.82 (d)	0.82 (d)	0.82 (d)	0.82 (d)
	27-H	0.84 (d)	0.84 (d)	0.84 (d)	0.84 (d)
	28-H	0.92 (d)	0.91 (d)	0.92 (d)	0.92 (d)
E.i.m.s.[b]	M^+	396,50%	396,67%	396,100%	396,47%
	M^+−Me	381,28%	381,1.0%	381,3%	381,7%
	M^+−Me−H_2O	363,0.0	363,100%	363,78%	363,7%
	M^+−SC	—	271,22%	271,16%	271,100%
	M^+−SC−H	270,100%	—	—	—
	M^+−SC−H_2O	255,30%	—	—	—
	M^+−SC−H_2 −2H	253,6.0%	253,65%	253,37%	253,69%
G.l.c.[c]	RRT$_c$ SE-30	1.12	1.15	1.22	1.20
	RRT$_c$ OV-17	1.26	1.25	1.38	1.33
	RRT$_c$ SP-1000	1.33	1.24	1.56	1.41
RP h.p.l.c.[d]	α_c 96% aq. MeOH	0.69	0.71	0.76	0.71
	α_c 90% aq. MeOH	0.87	0.89	—	0.91
T.l.c.[e]	R_f (Ad)	0.33	0.33	0.33	0.33
	R_f (RP)	0.40	0.40	0.30	0.30
	U.v.[f] λ (nm)	λ 249 ε 20 025	End adsorption	λ 262; 271; 282; 293 ε 7 983, 11 400, 11 900 6 900	λ 262; 253; 244 ε 19 048, 27 976, 26 180
Vinyl absorption in ^{1}H n.m.r.		5.2—5.4 (15-, 22-, 23-H, m)	5.43 (6-H, d) 5.20 (22-, 23-H, m)	5.20 (22-, 23-H, m) 5.37 (6-H, m) 5.58 (7-H, m)	6.13 (7-H, d) 5.12—5.37 (6-, 22-, 23-H, m)

[a] ^{1}H N.m.r.: Nicolet 200 MHz spectrometer operating at ambient temperature in the Fourier transform mode, solvent $CDCl_3$, δ values relative to Me_4Si. [b] E.i.m.s.: 70 eV, VC micromass (70/70) mass spectrometer, ion source, 150 °C. SC = side chain. [c] G.l.c. Varian 1440, flame ionisation adsorption detector, 3% SE and 3% OV-17 packed columns on Gas Chrom Q 100/120 mesh, 1.8 m by 2 mm internal diameter, and 1% SP-1000 on Supelcoport 100/120 mesh, 1.8 m by 2 mm, ionisation detector temperature 310 °C and oven temp. 240 °C, flow rate of He (carrier) 30 ml min^{-1}. RRT$_c$ = relative retention time. [d] H.p.l.c. Altex column packed with Ultrasphere ODS (5 μm), 4.6 mm internal diameter by 250 mm. Flow rate, 1.6 ml min^{-1}. Detector, 205 nm. [e] Ad = Adsorption, RP = reverse phase. Ad-t.l.c. benzene–diethyl ether (9 : 1) as developing solvent. RP-t.l.c. acetone–H_2O (47 : 3) as developing solvent. [f] U.v. sample dissolved in ethanol.

detector. The three sterols were observed to elute a couple of minutes earlier than ergosterol in a ratio of 4 : 3 : 1 possessing absorption spectra spectra with $\lambda_{max.}$ at 248, 253 nm, and end absorption, respectively. Because one of these three compounds may have been a novel sterol, *i.e.*, that sterol exhibiting $\lambda_{max.}$ 253 nm, we designed studies to elucidate their structures and biosynthetic relationships to ergosterol.

Sterols (**1**), (**2**), and (**4**) were easily separated from ergosterol (**3**) by reversed-phase h.p.l.c. eluted with 96% aq. MeOH and (**4**) and (**2**) were separated from (**1**) by carefully collecting the leading edge of the first peak and tail portion of the second peak of the two sterols, injected into the reversed-phase h.p.l.c. and eluted with 90% aq. MeOH. Finally, (**2**) and (**4**) were separated from each other by reversed-phase t.l.c. (Table 1). While we independently separated the sterol mixtures as their C-3 acetates using $AgNO_3$-impregnated Silica Gel G plates, we also developed separation techniques which obviated potential isomerization of the conjugated double-bond systems.

Comparison of the electron-impact mass spectra (e.i.m.s.) ^{1}H n.m.r. and u.v. spectra with the reported literature[1—6,8,11,12] confirmed the identity of the four sterols. While the u.v. and vinyl absorption in ^{1}H n.m.r. are very helpful in characterizing the nuclear double bond status (position, heteroannular, or homoannular, *etc.*), additional diagnostic structural information is gained with the aid of e.i.m.s., although much caution should be exercised in its use. We have observed that the fragmentation pattern of ergostatrienes is affected by the temperature of the ion source. For instance, at <280 °C (ion source) the spectrum for (**3**) is similar to that for (**2**) obtained at an ion source temperature of 150 °C and (**1**) and (**4**) may also appear similar to (**3**) depending on the precise operating conditions of the mass spectrometer. Thus an examination of the fungal sterol composition simply by g.l.c.–m.s. may not always be sufficient to discriminate the presence of minor ergostatrienes in a g.l.c. peak which otherwise cochromatographs with authentic (**3**). For this reason, it is conceivable (**4**) may be more widely occurring than is currently assumed.

In order to confirm that (**4**) was biosynthesized by *G. fujikuroi*, [2-^{14}C]acetate was fed to the fungus.[10] The SE fraction, obtained following acetone extraction of the mycelia and t.l.c. of the acetone extract, was diluted with 200 μg of non-radioactive (**4**) and saponified. The resultant free sterols,

Table 2. Metabolic interrelationships of ergostatrienes endogenous to *G. fujikuroi*.

Substrate	Activity fed to fungus	Total uptake of label which accumulated into mycelia–acetone extract	Activity recovered in ergosterol/%	Ratio of activity in free sterol and steryl ester fractions
[^{3}H]-(**1**)	1.1×10^7 d.p.m./500 μg	4.0×10^6 d.p.m.	6.0	80 : 20
[^{14}C]-(**2**)	3.9×10^4 d.p.m./118 μg	7.12×10^3 d.p.m.	15.4	50 : 50
[^{14}C]-(**3**)	4.47×10^5 d.p.m./534 μg	1.12×10^5 d.p.m.	59.0	97 : 3
[^{3}H]-(**4**)	4.0×10^6 d.p.m./450 μg	9.4×10^5 d.p.m.	0.00	97 : 3

obtained by adsorption t.l.c., were separated by reversed-phase h.p.l.c. and the material at α_C 0.69—0.71 collected for further purification. To this material [initial ^{14}C-activity; 6630 disintegrations per minute (d.p.m.)], we added freshly h.p.l.c. purified [2,4-$^3H_2$]-(**4**) (synthesized by base-exchange using ^3H_2O, as described[1,2]) producing a labelled sample with a ^{3}H/^{14}C ratio of 2.05/1. Additional chromatographic steps (Table 1) devised to produce pure (**4**), resulted in a radioactive sterol with a ^{3}H/^{14}C ratio 2.3/1. Isotopic dilution with 13.0 mg of unlabelled (**4**) followed by recrystallizations from MeOH, acetone–H_2O, C_6H_6–CH_2Cl_2 produced materials having similar specific activities: ^{3}H 520, ^{14}C 230 d.p.m. mg^{-1} and the activity remained in a constant ratio of ^{3}H/^{14}C 2.26/1 through each of the three recrystallizations. The maintenance of radiospecificity in the ^{3}H/^{14}C ratio clearly shows that no minor sterol was contaminating our sample of (**4**).

In the next series of experiments we chemically synthesized radiolabelled [2,4-$^3H_2$]-(**1**) and -(**4**) and produced [^{14}C]-(**2**) and -(**3**) through incubations with [2-^{14}C]acetate and *G. fujikuroi*.

As shown in Table 2, (**1**) and (**2**) were metabolized to (**3**). Ergosterol was not converted into either (**1**), (**2**), or (**4**). The inability to convert (**3**) into (**2**) indicates that the isomerase,[7] if capable of being bi-directional, must have precise substrate specificities or be compartmentalized in such a way that in *G. fujikuroi* ergosterol is not utilizable for the $\Delta^7 \rightarrow \Delta^8$ isomerisation.

The biosynthetic origin of (**4**) remains enigmatic, although in light of [2,4-$^3H_2$]-ergosta-8(14)-monoene-3β-ol metabolism by *G. fujikuroi* in high yield to (**3**) but not (**4**),[13] it would appear that this fungus lacks a Δ^6-desaturase similar to other organisms. We suspect that its intermediate is a Δ^7-14α-methylsterol which is demethylated at C-14 and the resultant prototropic migration is directed toward the B/C rings [*i.e.*, to produce a $\Delta^{6,(7),8(14)}$-sterol], rather than to the C/D rings [*i.e.*, to produce a $\Delta^{7(8),14(15)}$-sterol].

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