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Cycloartenol-derived sterol biosynthesis in the Peronosporales

(Pythiaceae/*Phytophthora*/*Pythium*/*Peronophythora*)

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ABSTRACT **Selected species of the order Peronosporales, which are unable to epoxidize squalene and thus synthesize sterols, are able to metabolize exogenous cycloartenol to lanosterol and in some organisms to fucosterol, ergosterol, and cholesterol. Lanosterol was less effectively utilized but some ergosterol and cholesterol were yielded. Fucosterol was very efficiently metabolized by most species to ergosterol, Δ^7-ergostenol, Δ^5-ergostenol, cholestanol, and cholesterol. Several unknown sterols were observed in most trials. These data suggest a vestigial sterol synthetic pathway derived from cycloartenol, followed by possible isomerization to lanosterol and then to other sterols.**

It has been established that species of *Pythium* and *Phytophthora* require exogenous sterols for sexual reproduction (1, 2). These organisms are unable to synthesize sterols because of a missing squalene epoxidase (3). Metabolism of exogenous sterols has been observed in *Phytophthora cactorum* by Langcake (4), who reported possible conversion of lanosterol to cholesterol. Nes and Patterson (5) reported that *Phyt. cactorum* was unable to transform cycloartenol or lanosterol to other sterols. Conversion of Δ^7 and $\Delta^{5,7}$ sterols to Δ^5 sterols has been reported by Elliott and Knights (6) in *Phyt. cactorum*, which additionally synthesizes cholesteryl esters, cholesteryl glucosides, and acyl sterol glycosides (7, 8). Other than the above, sterol conversions in *Phytophthora* or *Pythium* have, in general, not been demonstrated in detail.

Lagenidium giganteum is another Oomycete, but of the Lagenidiales, that requires exogenous sterols to produce zoospores (9). Warner and Domnas (10) have shown that this organism converts cycloartenol, but not lanosterol, to cholesterol. Because *Lagenidium* species are able to metabolize cycloartenol, it was of considerable interest to observe if this compound could be metabolized by selected species of the Peronosporales. This article reports our observations.

MATERIALS AND METHODS

Microorganisms, Maintenance, and Growth. *Phyt. cinnamomi*, *Phyt. infestans*, and *Pythium debaryanum* were obtained from Carolina Biological (Burlington, NC). *Phyt. cryptogea* and *Phyt. parasitica* var. *nicotianae* were obtained courtesy of J. D. Nelson, Jr. (Olin Research Center, New Haven, CT). *Phyt. cactorum* (no. 16693) and *Peronophythora litchii* (no. 34595) were obtained from American Type Culture Collection (Rockville, MD). Stock cultures were maintained on 2% agar slants with the defined medium of Elliott and Knights (7).

Inoculation and growth were as described (10) with the defined medium of Elliott and Knights (7). Five replicates were prepared for at least three different growth trials.

Sterols. Lanosterol ($\Delta^{8,24}$-lanostadienol) and fucosterol ($\Delta^{5,24(28)E}$-stigmastadienol) were purchased from Research Plus Steroid Laboratories (Denville, NJ). Cycloartenol (9β, 19-*cyclo*-Δ^{24}-lanostenol) was prepared by the method of Nath (11) from jackfruit supplied through the courtesy of Y. Sagawa (Lyon Arboretum, University of Hawaii at Manoa). Crude cycloartenol was purified by repeated column chromatography (12) followed by preparative reverse-phase HPLC (elution with methanol/H_2O, 96:4; Waters preparation LC500A; elution rate 250 ml/min). The cycloartenol and fucosterol were found to be essentially >99.9% pure as assayed by gas/liquid chromatography (see below), but lanosterol contained 35% dihydrolanosterol. Sterols were added to the broth (1 mg/100 ml) in $CHCl_3$ along with 1 ml of 2% aqueous Tween 80 and autoclaved.

Sterol Isolation and Identification. Vegetative mycelia collected after 7 days' growth were treated as described (10) and the unsaponifiable lipid fraction was obtained. The sterols were separated on HPLC with a Waters instrument by using a 10-μm C_{18} column (4.6 × 250 mm). Fractions were eluted with methanol/H_2O (96:4) at 2 ml/min flow with UV detection at 210 nm. Sterols were identified by gas/liquid chromatography on 3% SE-30 and 1% QF-1 with a Packard 417 FID chromatograph as described (10). Confirmation of identification was obtained with gas chromatography/electron impact mass spectroscopy (70 eV) with a Finnegan 4023 instrument. A 15-m flexible fused silica capillary coated with OV-101 was programmed from 160 to 260°C at 10°C/min with an initial hold of 3 min.

RESULTS

No sterols were found in control organisms to the limit of detection (0.0001%). Uninoculated control cultures yielded a stoichiometric recovery of the administered sterols.

Sterols Isolated After Administration of Cycloartenol. Table 1 illustrates the substances identified in the organisms examined. Clear evidence was obtained for the conversion of cycloartenol to lanosterol; however, the conversion efficiency—defined as the percent of sterol taken up that was converted—varied from organism to organism and ranged from 2% to 40%. Total neutral sterols were typically 5–10 μg/mg of dry weight here and below. Three of the five *Phytophthora* species yielded cholesterol. *Phyt. cinnamomi* also yielded 14α-methyl-$\Delta^{5,8}$-cholestadienol and traces of fucosterol. Desmosterol was found in *Phyt. cinnamomi*, *Phyt. cryptogea*, and in *Peronophythora litchii*. *Phyt. parasitica*, *Phyt. cactorum*, *Pythium debaryanum*, and *Peron. litchii* also synthesized ergosterol. Choles-

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Table 1. Sterols isolated from Peronosporales after growth with cycloartenol

Organism	Sterols isolated*	% composition
Phytophthora cactorum	Cycloartenol	96
	Lanosterol	1
	Ergosterol	1
	Cholesterol	1
	$\Delta^{5,22}$-Stigmastadienol	1
	M^+ = 426 (1.27)	NQ
Phyt. cinnamomi	Cycloartenol	60
	Lanosterol	30
	Cholesterol	5
	14α-Methyl-$\Delta^{5,8}$-cholestadienol	5
	Fucosterol	NQ
	Desmosterol	NQ
	M^+ = 400 (1.00)	NQ
	M^+ = 440 (1.28)	NQ
Phyt. cryptogea	Cycloartenol	98
	Lanosterol	1
	Desmosterol	1
	M^+ = 408 (1.02)	NQ
	M^+ = 424 (1.11)	NQ
	M^+ = 424 (1.14)	NQ
Phyt. infestans	Cycloartenol	98
	Lanosterol	1
	Cholesterol	1
	M^+ = 424 (1.12)	NQ
Phyt. parasitica	Cycloartenol	94
	Lanosterol	2
	Ergosterol	2
	Cholestanol	1
	Cholesterol	1
Peronophythora litchii	Cycloartenol	97
	Lanosterol	1
	M^+ = 426 (1.27)	1
	Cholesterol	1
	Ergosterol	NQ
	Desmosterol	NQ
Pythium debaryanum	Cycloartenol	95
	Lanosterol	5
	Cholesterol	NQ
	Desmosterol	NQ

Fungi were grown and sterols analyzed as described in *Materials and Methods*. The area normalization for the percent composition was determined with the SE-30 column. NQ = detected but not at quantifiable levels.

* Unknown sterols are designated by their molecular ion. Values in parentheses refer to retention time relative to cholesterol on OV-101.

tanol was identified in *Phyt. parasitica*. $\Delta^{5,22}$-Stigmastadienol was present in *Phyt. cactorum*.

A considerable number of unknown sterols was found from M^+ 398 to M^+ 428, of which M^+ 400, 424, and 426 were most frequently encountered. These unknowns were considered sterols only if M^+, $(M-CH_3)^+$, $(M-H_2O)^+$, and $(M-[CH_3+H_2O])^+$ were discernible and the relative retention times were appropriate.

Sterols Isolated After Administration of Lanosterol. All organisms transformed lanosterol to some extent with a conversion efficiency ranging from 1% to 7% (Table 2). Despite this low rate of utilization, the surprising presence of ergosterol was easily detected in *Phyt. cinnamomi, Phyt. parasitica, Phyt. cactorum,* and *Pyth. debaryanum,* with trace quantities in *Peron. litchii*. The last three organisms also produced cholesterol as did *Phyt. infestans*. Trace amounts of Δ^5-stigmastenol were found

Table 2. Sterols isolated from Peronosporales after growth with lanosterol

Organism	Sterols isolated	% composition
Phytophthora cactorum	Lanosterol/dihydrolanosterol	93
	Ergosterol	3
	Cholesterol	2
	Δ^5-Ergostenol	1
	M^+ = 424 (1.11)	1
Phyt. cinnamomi	Lanosterol/dihydrolanosterol	96
	Ergosterol	4
	M^+ = 398 (1.00)	NQ
	M^+ = 424 (1.11)	NQ
	M^+ = 424 (1.04)	NQ
	M^+ = 426 (1.07)	NQ
Phyt. cryptogea	Lanosterol/dihydrolanosterol	99
	M^+ = 394 (1.00)	1
	M^+ = 400 (1.00)	NQ
Phyt. infestans	Lanosterol/dihydrolanosterol	95
	Cholesterol	5
Phyt. parasitica	Lanosterol/dihydrolanosterol	97
	M^+ = 426 (1.07)	2
	Ergosterol	1
	Δ^5-Stigmastanol	NQ
	M^+ = 440 (1.28)	NQ
Peronophythora litchii	Lanosterol/dihydrolanosterol	99
	Cholesterol	1
	Ergosterol	NQ
	M^+ = 424 (1.14)	NQ
	M^+ = 426 (1.07)	NQ
Pythium debaryanum	Lanosterol/dihydrolanosterol	98
	Ergosterol	1
	Cholesterol	1
	M^+ = 426 (1.07)	NQ

Legend and footnote as in Table 1.

in *Phyt. parasitica. Phyt. cactorum* exhibited Δ^5-ergostenol. Again a considerable number of unknown sterols was observed, ranging from M^+ 394 to M^+ 440.

Sterols Isolated After Administration of Fucosterol. All organisms utilized fucosterol in efficiencies ranging from 3% to 62%, and all produced cholesterol (Table 3). *Phyt. cinnamomi* produced ergosterol (38%) and Δ^7-ergostenol (12%), which was also found in *Phyt. parasitica, Pyth. debaryanum,* and *Peron. litchii*. Cholestanol was found in significant quantities (10%) in *Phyt. cryptogea* and *Phyt. parasitica*. Numerous unidentified sterols ranging from M^+ 394 to M^+ 428 were present.

DISCUSSION

The Oomycetes and Hyphochytriomycetes are considered by many mycologists to have a phylogenetic origin different from all other fungi, in view of a variety of morphological and biochemical parameters (13). All other fungal classes cyclize 2,3-oxidosqualene to lanosterol (3, 14, 15), whereas algae and land plants (16, 17), *Lagenidium* species (10), and *Saprolegnia* species (18) of the Oomycetes cyclize this compound to cycloartenol. The metabolism of cycloartenol to lanosterol and other sterols by selected species of the Peronosporales indicates that the sterol synthetic pathway past the 2,3-oxidosqualene cyclase

Table 3. Sterols isolated from Peronosporales after growth with fucosterol

Organism	Sterols isolated	% composition
Phytophthora cactorum	Fucosterol	83
	M‡ = 394 (1.00)	12
	M‡ = 410 (1.22)	4
	Δ^7-Ergostenol	1
	Cholesterol	NQ
	Desmosterol	NQ
Phyt. cinnamomi	Ergosterol	38
	Fucosterol	30
	Δ^7-Ergostenol	12
	M‡ = 394 (1.00)	10
	Δ^5-Ergostenol	5
	Cholesterol	3
	M‡ = 410 (1.22)	2
Phyt. cryptogea	Cholesterol	50
	Fucosterol	40
	Cholestanol	10
Phyt. infestans	Fucosterol	91
	Δ^7-Ergostenol	9
	Cholesterol	NQ
	Desmosterol	NQ
	Cholestanol	NQ
Phyt. parasitica	Fucosterol	94
	Cholestanol	2
	M‡ = 410 (1.22)	2
	M‡ = 428 (1.22)	2
	Ergosterol	NQ
	Δ^5-Ergostenol	NQ
	Δ^7-Ergostenol	NQ
	Cholesterol	NQ
	Lathosterol	NQ
Peronophythora litchii	Fucosterol	97
	Δ^5-Ergostenol	1
	Δ^7-Ergostenol	1
	Cholesterol	1
Pythium debaryanum	Fucosterol	94
	M‡ = 410 (1.22)	5
	Cholesterol	1
	Δ^7-Ergostenol	NQ
	Desmosterol	NQ
	M‡ = 394 (1.12)	NQ
	M‡ = 424 (1.18)	NQ
	M‡ = 428 (1.22)	NQ

Legend and footnote as in Table 1.

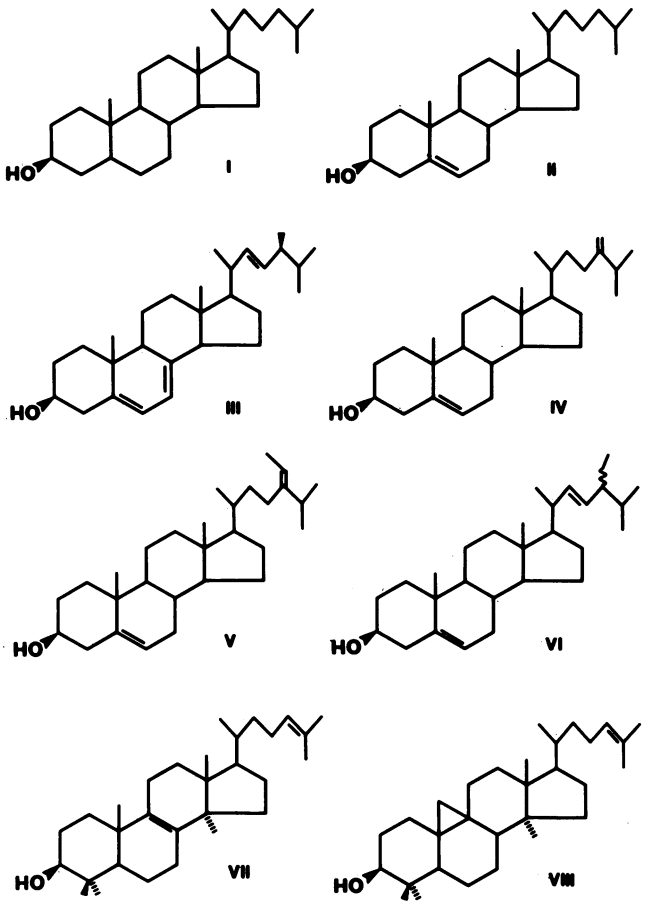

FIG. 1. Structures of cholestanol (I), cholesterol (II), ergosterol (III), 24-methylene cholesterol (IV), fucosterol (V), stigmasta-5,22-dienol (VI), lanosterol (VII), and cycloartenol (VIII).

is intact and that cycloartenol may have been the product of cyclization by their sterol-synthesizing ancestors. The structures of several of the sterols described in this text are shown in Fig. 1.

We have attempted to reconcile our findings with those of Nes and Patterson (5), who were unable to demonstrate metabolism of cycloartenol or lanosterol. Their organism was grown under static conditions for 18 days with 50 ml of media per culture flask, yielding about 110 mg of dry weight per culture. We used twice their volume of media with growth for 7 days on a Gyrotory shaker, yielding ≈500 mg of dry weight per culture. Furthermore, we solubilized our sterols with Tween 80, which is mildly stimulatory of growth (19), rather than the ethanol which they employed. Thus, our organisms were under conditions of more active growth, which may account for the difference in our findings.

Fucosterol, a common Oomycete sterol (1) and a product of cycloartenol administration to *Phyt. cinnamomi*, was effectively utilized by all of the organisms, with ergosterol, Δ^7-ergostenol, desmosterol, and other sterols produced. These results suggest the presence of a native metabolic competence to metabolize fucosterol. The occurrence of ergosterol was unexpected, as it had previously been observed in only one other Oomycete, *Zoophagus insidians* (20). Because fucosterol was not a product of lanosterol administration it may presumably derive *via* a pathway from cycloartenol that excludes lanosterol, even though lanosterol can be produced from cycloartenol. It appears that two distinct pathways from cycloartenol exist, based on this evidence and the presence of various 24-methyl and 24-ethyl sterol metabolites which are difficult to reconcile with a single pathway. Previous studies with *Lagenidium* species (10) showed that cycloartenol, but not lanosterol, produced fucosterol and cholesterol. Conversely, *Z. insidians* produces ergosterol along with trace amounts of lanosterol and 24-methyl sterols (20). We suggest that *Lagenidium* and *Zoophagus* possess differentially enhanced branches of the same overall cycloartenol-derived pathway observed in this study and that these branches function at a more equal level of efficiency in the Peronosporales. A scheme to encompass these concepts is shown in Fig. 2, with a dealkylation of 24-ethyl sterols postulated to occur as has been observed for insects (21). A branched pathway in which lanosterol may be bypassed metabolically has also been described in plants (22).

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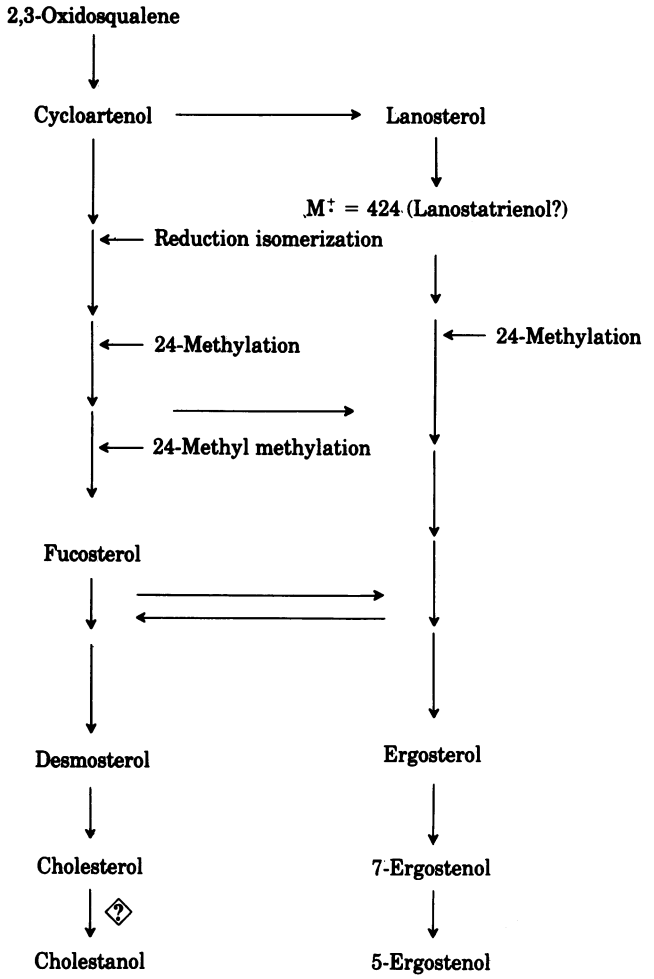

FIG. 2. Suggested pathways of sterol synthesis in the Peronosporales.

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