Side-Chain Structural Requirements for Sterol-Induced Regulation of *Phytophthora cactorum* Physiology

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ABSTRACT

The influence of cholesterol, (E)-17(20)-dehydrocholesterol, sitosterol, (Z)-17(20)-dehydrocholesterol and 20-isocholesterol on growth and sexual morphogenesis in *Phytophthora cactorum* has been examined. Optimal growth-response and production of oospores occurred with the 3 former sterols, which possessed "right-handed" side chains (C22 *trans*-oriented to C13). Abnormal hyphae and aborted oospores were evident in mycelia cultured with sterols having side chains with "left-handed" structures, i.e., 20-isocholesterol and (Z)-17(20)-dehydrocholesterol. The induction of the sexual cycle lacked a selectivity for stereochemistry in the side chain. The results are interpreted to imply that fungal recognition of the sterol molecule in the reproductive phase of the life cycle is of 2 types: one involves discrimination of stereochemical features of the sterol side chain (oospore production); in the other, no functional significance can be attributed to conformation or configuration of side chain moieties (sexual structure induction). Growth response to dietary sterol seems to fall into the former category.

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INTRODUCTION

The ultimate tetracyclic products, e.g., 4,4,14-tris-desmethylsterols, of 2,3-oxidosqualene cyclization that occur as membrane components, are sterols that possess a 20α-H atom and a side chain that presumably orients functionally as the right-handed skew conformer (1). Fungi (1-4), bacteria (5-7) and insects (8,9) with a nutritional dependence on polycyclic isopentenoids (steroids and triterpenoids) for growth and reproduction have been useful model systems to assess the similarities (and differences) in the biochemical and physiological roles of these molecules in otherwise evolutionarily divergent organisms. *Phytophthora cactorum*, which causes a collar rot of various crop roots, is especially attractive as an organism with which to explore the significance of sterol function. Despite the fungus's failure to epoxidize squalene (10,11), dietary sterols available from its host or the culture medium (4) are accumulated by mycelial membranes (12,13) affecting growth (14,15) and biochemical properties (3,16-18). Reproduction can also be "turned-on," resulting in the production of numerous oospores (19).

The purpose of the present communication is to report the effect of several sterols that are stereochemically modified, producing "right- and left-handed" side chains (1), for their ability to concurrently stimulate growth and induce oospores in *P. cactorum*. In order to form a basis for the structure-activity comparisons, detailed studies of uptake, derivatization and physiological parameters, with cholesterol as a standard, were made first. Previous investigations with the C20 stereoisomers of cholesterol in supporting growth of anaerobic yeast (20-22), metabolism by a protozoan (23), inhibition of hepatic cholesterol synthesis in a mammal (24) and lipid vessicle formation (25) demonstrated that the extent to which the side chain is recognized depends on the biological and physicochemical systems involved. In the present study we observed that the ability for the fungus to discriminate between the various synthetic and naturally occurring side chains is significant to the life cycle of the pathogen. In contrast, however, to inferences in the literature (26,27), we now find that the kinds of recognition of the sterol by *P. cactorum* are not as found in *Achyla*, a related Oomycete, in the induction of the sexual cycle. Alternatively, some similarities in the sterol requirements for membrane structure are implied.

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MATERIALS AND METHODS

Culture Methods

The test organism, *P. cactorum* (strain 51-22), obtained from the U. C. Berkeley fungal collection, was grown on a synthetic sucrose-asparagine medium as described by Elliott (26) and modified according to Nes et al. (14). The method for quantitating the various sexual reproductive structures has been described in the literature (19,26,30). Two stock cultures were routinely maintained at room temperature: one set was maintained on clarified V8 juice solidified by the addition of

Difco agar (20 g/l) and a second set was maintained on agar-supplemented synthetic medium. The only difference in the medium used for the experimental cultures with that of the second set was the addition of sterol dispersed in ethanol. Difco agar was recently shown to contain trace levels of cholesterol (28,29). The mycelia, originally cultured on the V8 juice–agar medium and producing an orange colored mat with no significant aerial hyphae, were transferred to a synthetic medium supplemented with agar. Every 2 weeks the fungus was transferred (5 mm plug) to fresh, agar-supplemented synthetic medium to which no additional sterol had been added. After ca. 2 transfers, the mycelia were white with aerial mycelium. This mat form served as the inoculum source for the various sterol supplementation experiments. When the mycelium was serially transferred to synthetic agar media (containing only trace sterol), the ability of the fungus to produce oospores in response to cholesterol supplementation diminished (4). Thus, every 6 months we initiate new synthetic, agar-supplemented stock cultures from the V8-cultures. Sterols (10 μg/ml of medium) were added as an ethanolic solution (10 μl or 2 μl/ml of medium, depending on the treatment) to the agar-supplemented synthetic media as the agar was solidifying. Ethanol at 2 μl/ml had no effect on growth or reproduction of cultures grown on agar. The higher level of ethanol (10 μl/ml) had no observable effect on reproduction or hyphal extension. However, as recently reported (31), dry wt of the fungus was increased by ca. 50% with 10 μl/ml ethanol. This ethanol effect was independent of the addition of sterol (within the concentration range tested) to the medium. No apparent synergistic or additive effects resulted from sterol-ethanol combinations. The effect that the greater amount of ethanol has on dry wt production may be related to the respiratory competency of the mitochondria analogous to that described in yeast (32). Analogous effects of high levels of ethanol (10 μl/10 ml of medium) supplied to mycelia cultured on liquid media are not apparent; in fact this level inhibits their growth (Poley and Nes, unpublished data). Also we have found that 0.5 μg of sterol/ml of medium is sufficient to stimulate maximal growth of mats cultured in synthetic liquid medium (Nes and Poley, unpublished data). We preferred to use 10 μg/ml of sterol in the present set of experiments because this level produced maximal oospores numbers. As the amount of sterol in the media decreases a corresponding decrease in the number of oospores is observed (Nes and Poley, unpublished data).

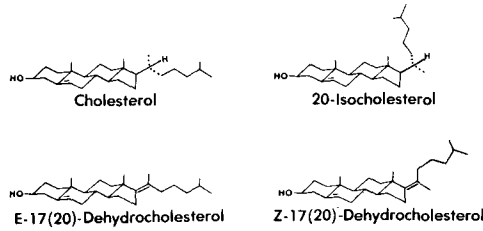

FIG. 1. Structures of some of the sterols incubated with *P. cactorum*.

Chemicals

[4-^{14}C] Cholesterol (59.4 mCi/m Mol) was purchased from New England Nuclear, Boston, MA. Cholesterol and sitosterol (24α-ethylcholesterol) were purchased from Applied Science Labs, State College, PA, and recrystallized from ethanol. The sitosterol contained a small percentage of 24-methylcholesterol. This contaminant was removed by chromatographing the commercial sitosterol on LH-20 Sephadex, developed isocratically with 5% MeOH in hexane. 20-Isocholesterol (also referred to as 20-epicholesterol), (Z)- and (E)-17(20)-dehydrocholesterol were the gifts of Dr. W. R. Nes. They had been synthesized according to established methods (33). Their structures are shown in Figure 1.

Lipid Extraction and Analysis of Sterols

The mycelia in each petri dish (5 dishes per structure-activity treatment) were recovered from the agar at each harvest (34). The mycelia were dried in vaccuo in an Abderhalden apparatus and then weighed, ground to a powder and extracted in a Soxhlet apparatus with refluxed acetone for 18 hr. In order to assess the derivatization of [^{14}C] cholesterol by the mycelia, the total lipid extract (TLE) from each harvest was chromatographed by TLC according to Nes et al. (34). Zones matching free sterols, sterylesters and sterylglycosides were scraped from the plate into scintillation vials containing POPOP cocktail (5 ml) and the radioactivity determined.

Sterols, reisolated from the fungus (without saponification) by thin layer chromatography (TLC) (34), were chromatographed on 3 packed gas liquid chromatographic (GLC) columns having different polarities. The retention times relative to cholesterol on 3% SE-30, 3% OV-17, and 1% SP-1000 packed columns (operated isothermally at 235°, 235° and 255°, respectively) for the 5 test sterols (≤ 99% pure by GLC) were: stiosterol–1.61, 1.68, 1.32; 20-isocholesterol–0.91, 0.89, 0.89; (E)-17(20)-dehydrocholesterol–0.93, 1.00, 0.98; and (Z)-

17(20)-dehydrocholesterol–0.87, 0.91, 0.92. We employed the 3 packed columns routinely because the EI-MS of the pairs, 20-isocholesterol and cholesterol, and (E)- and (Z)-17(20)-dehydrocholesterol are very similar (33). Furthermore, long-chain fatty alcohols were found to cochromatograph with the sterols on some TLC and GLC systems (29)–this can affect sterol quantitation. We used GLC-MS (GLC column–3% OV-17) to confirm the identities of the sterols found eluting in at least one GLC system used for routine analysis.

RESULTS AND DISCUSSION

Growth and Reproduction with Cholesterol

Although a great deal of information is available on sterol affecting both growth and reproduction of *P. cactorum* (for a recent review, cf. 19), the sequence of physiological events that result from the addition of cholesterol to the media has not been followed from the initiation of growth to the formation of oospores. Because we have been unable to produce oospores or oogonia in the liquid synthetic media over a 28-day incubation period (20° incubation temp) with 10 ppm of cholesterol, we chose to use the agar solidified synthetic media–a system previously shown to permit cholesterol to induce oospore production (28, 30). As shown in Figure 2, cholesterol initiates and maintains optimal growth, measured as changes in hyphal extension and dry weight. This "sparking" (40) of growth, in which the length of the lag phase is reduced by sterol supplementation, has recently been observed in yeast auxotrophic for sterols (40). The next event (Fig. 3) is the formation of the female sexual structures (oogonia), followed by fertilization and maturation of the latter into double-walled oospores. No consequential reproduction occurs in the absence of sterol (data not shown). In some of our experiments the control produces a few oogonia that variably mature into oospores. This, however, is attributable to the trace levels of sterol in the agar (29).

Derivatization of Cholesterol

Previous investigations (35,36) that examined sterol derivatization in *P. cactorum* were conducted under conditions in which no sexual reproduction occurred. In order to gain a more complete picture we examined this problem with cholesterol under culture conditions where multiple physiological processes could similarly be monitored. For the radiolabeled sterol experiment, [4-^{14}C]-cholesterol (3.6×10^6 cpm/1.8 mg cholesterol), as an ethanolic

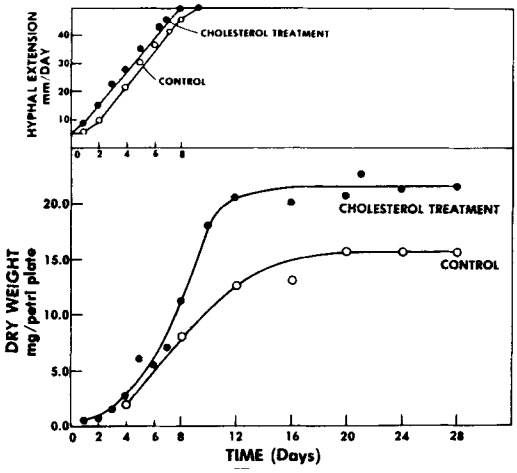

FIG. 2. Growth curves (top–growth measured as changes in hyphal extension; bottom–growth measured as changes in mycelial dry weight) of *P. cactorum* cultured on solid media at 20° in the dark.

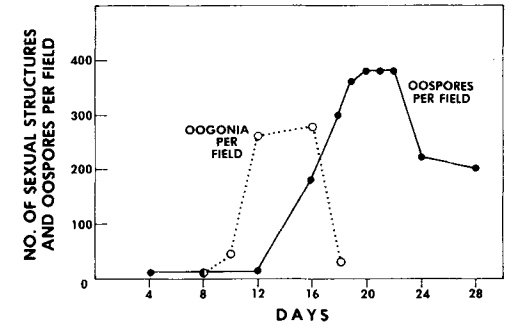

FIG. 3. Sequence of reproductive events exhibited by *P. cactorum* that can be readily observed microscopically and quantitated. Under these test conditions male sexual organs are not readily apparent, nor are the asexual zoospores.

solution, was distributed equally (5 ml aliquot) into each of 36 petri dishes, each of which contained 10 μg nonradioactive cholesterol and 2 μl ethanol per ml of medium. The first harvest (day one, post inoculation) contained the mycelia from 10 petri dishes. The second harvest (days 4, 6, 7-10, 14, 16, 20, 24, 28) contained mycelia from 2 petri dishes. Insufficient mass was available in the 24-hr period before the first harvest to obtain earlier data points. When the TLE of each of the mycelia harvested during the 28-day period was examined by TLC radioscanning and by comparing the radioactive counts in the 3 zones corresponding to free, esterified and glycosylated sterols, no significant differences in time were apparent in the ratio (ca. 72:26:2) of these molecular forms. In a separate experiment, cultures (ca. 5 per

treatment) with 10 ppm cholesterol were harvested at 12, 15, 21 and 28 days. The amount of free sterol in the mycelium was ca. .01% of the dry weight in each of the 4 harvests. Thus, no change occurred in the level of free or derivatized cholesterol in the mycelia during growth or reproduction. These findings contrast with data obtained with fungi capable of de novo sterol synthesis where, on entering the stationary phase, the sterylester pool greatly increases in relation to the free sterol pool (32). Furthermore, our results agree with Hendrix (37,38) and do not confirm the suggestion of Elliott and Knights (35) that derivatization of free sterols controls reproduction. In another experiment (data not shown) the TLE from the combined mycelia (5 petri dishes) of a cholesterol treatment (10 ppm) was chromatographed by TLC. The TLC plate was divided into 15, 1 cm zones and each zone scraped and the material eluted from the plate with ether. Each of these zones was bioassayed for oospore production. The only zone that produced significant oospores was the zone corresponding to 4,4-desmethyl sterols. Although we and others (39) observe the occurrence of steroid metabolites (distinct from the sterylglycosides and sterylesters, therefore presumably autooxidation products) from radiolabeled sterol feeds with *P. cactorum,* these compounds, as they occur in the mycelium, have no obvious biological activity. Had derivatization of the steroid pool been a major element in the reproductive cycle, we would have expected a significant change in the free sterol-to-derivatized pool ratio before or at the onset of oospore production.

Structure-Growth Relationships

Growth response to sterol supplementation was measured in 2 ways: changes in hyphal extension and dry weight. Growth and mycelial membrane biogenesis of filamentous fungi, e.g., *Phytophthora,* unlike single-celled organisms, e.g., yeasts, occur by localized extension of hyphal tips (41,42). The addition of 4 of the 5 test sterols to the culture medium affected both parameters of mycelial growth. Each of the 5 test compounds were recovered from stationary phase cultures and their identities confirmed by GLC and GLC-MS (mass spectroscopy). Cholesterol was present in every mycelial extract. To ensure that cholesterol was a contaminant in those cultures other than the cholesterol treatment and not the product of metabolism, e.g., reduction of the Δ17(20)-bond, we incubated another set of synthetic liquid cultures with 10 ppm of each of the sterols. These cultures were maintained for 2 weeks in 50 ml synthetic media/250 ml flasks and inoculated with a mycelial homogenate. The homogenate was formed from cultures that had been grown for 2 weeks on a sterol-free synthetic media and seeded with mycelial fragments. The GLC analysis of the 4-desmethyl zone from each mycelial extract failed to detect cholesterol, except in the cholesterol treatment. Thus, the inability of some sterols to effect a stimulatory growth-response in *P. cactorum,* e.g., (Z)-17(20)-dehydrocholesterol, cannot be caused by its lack of accumulation by the mycelium. Morphological abberations of 6-day-old cultures treated with 20-isocholesterol and Z-17(20)-dehydrocholesterol were observed in the extent of hypal branching; many hyphae grew as a single tube from the implant with no branch at all. The radial growth measurement for the 20-isocholesterol treatment is somewhat misleading because this response could be interpreted to imply a stimulatory effect; rather, we suggest, this compound has a deleterious effect on the fungus. Comparable rate of growth (hyphal extension) and mycelial morphology similar to the cholesterol treatment (43) was observed for (E)-17(20)-dehydrocholesterol and sitosterol. The addition of 3 of the 5 test sterols to the culture media stimulated growth as measured by the dry weight (Table 1), whereas 4 of the test sterols were stimulatory as measured by the radial growth. The results show that the initiation and maintenance of optimal growth (measured by both parameters) is accomplished by the 3 "right-handed" sterols.

Structure-Reproduction Relationships

From the time-course growth and reproductive studies with cholesterol treatment (Figs. 2 and 3), we observed that maximal oospore production occurred on the twenty-first day following the inoculations. Thus, all quantitative comparisons were made at this time. As shown in Table 1, all 5 test sterols induced oogonia formation. The naturally occurring 4-desmethyl sterols, e.g., cholesterol and fucosterol, when supplied to *Achlya* as an exogenous hormonal supplement, fail to induce sexual structure formation. Apparently only specific polyoxygenated steroids, e.g., antheridiol and oogoniol, derived by endogenous metabolism of fucosterol, stimulate the antheridia and oogonia to form (27 and ref. cited therein).

When sitosterol was bioassayed in *P. cactorum* more oospores were formed than with cholesterol or (E)-17(20)-dehydrolesterol. On

TABLE 1

The Effect of Sterols on Sexual Reproduction and Vegetative Growth in *Phytophthora cactorum*

Sterol added	Oogonia[a] Mean SD	Aborted oogonia Mean SD	Oogonia with oospores Mean SD	Total	Mean[b] 21-day dry weight (mg) Mean	Mean 6-day diameter (mm) Mean SD Rate
Cholesterol	0[b]	0	335 ± 43	335	30.1	42.0 ± 0.66 [6 mm/day]
20-Isocholesterol	150 ± 31	200 ± 67	10 ± 6	345	20.3	42.5 ± 1.02 [6.5 mm/day]
E-17(20)-Dehydrocholesterol	0[b]	0	313 ± 24	323	29.8	45.0 ± 0.66 [5.8 mm/day]
Z-17(20)-Dehydrocholesterol	60 ± 23	5 ± 5	0	65	22.8	37.0 ± 0.66 [6.0 mm/day]
24α-Ethylcholesterol	0	0	521 ± 51	521	30.0	43.0 ± 0.66 [6.0 mm]
Control (sterol-free)	5	0	0	5	21.0	36.0 ± 0.66 [6.0 mm/day]

[a]Mean (with standard deviation–SD) oogonia and oospore count with *P. cactorum* for 4 radial transerts in each of 5 petri plates. Mycelia were cultured for 3 weeks in the dark on agar at 20°. Sterols were added at a level of 10 μg/ml dissolved in warm ethanol at 2 μl/ml (v/v).

[b]The contents of 5 petri dishes were pooled into one flask and the dry weight obtained following the removal of the agar.

[c]The diameter was determined each day for 6 days; from this information the rate was calculated.

the other hand, 20-isocholesterol and E-17(20)-dehydrocholesterol failed to produce oospores. Because we observed oogonia and structures that appeared to be aborted oospores, i.e., hollow cells rather than double-walled structures, the "left-handed" sterols were probably recognized in a deleterious fashion by the fungal receptor sites at some point. That certain Δ5-sterols induce production of oogonia which fail to mature into oospores, we conclude that formation of oogonia and oospores are controlled by different biochemical component(s) and these may differ from those observed in other Oomycetes, e.g., *Achlya* (19).

In conclusion, the molecular requirements of structure demonstrated in this study for initiating and maintaining growth are similar to previous microbial studies with singled-celled organisms (22). However, unlike *Achyla* (44), where the induction of the female sex organs is influenced by the stereochemistry of the side chain (45), *P. cactorum* may use either of the "right- and left-handed" sterols for induction of the oogonia. The production of functional oospores, however, requires "right" handed sterols. Sterol derivatization appears to precede the formation of oogonia and no quantitative changes in the proportion of free sterol to esters or glycoside occur during oospore production. Interestingly, no steroid hormones have been implicated in oospore production, only in the induction of the male and female gametangia. Whereas the induction process may involve sterol feedback (19) on enzymes controlling the so-called "α-hormone" (46), oospore production itself appears to be regulated by the fitness of the sterol as a component of the maturing oogonia membranes, i.e., the developing double wall (28), rather than being metabolized to an oxygenated steroid hormone. The extent to which sterol-induced alterations in the mycelial membranes effect the oogonia maturation process requires further study.

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