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.


INTRODUCTION

The ultimate tetracyclic products, e.g., 4,4,14-tris-desmethylerol, 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 (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 vesicle 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.

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).

**Chemicals**

[4-1^4C] 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 vacuo 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 [14C] 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 POPPOP 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)-