Oomycetes such as Phytophthora and Pythium are among the few eukaryotes which lack a completed sterol pathway. These pythiaceous fungi synthesize squalene but are unable to epoxidize the hydrocarbon (9). Although species of Phytophthora and Pythium can grow vegetatively in the absence of dietary sterol (1, 2, 5, 10), the acquisition of sterol from an exogenous source is obligatory for the induction of sexual reproduction. Recently, steroids have been shown to be the chemical agents responsible for the induction of the sexual cycle in Achlya (12, 22).

Elliott and co-workers (3, 7) have examined the effect of sterol structure on sexual reproduction and reported that the presence of unsaturation in ring B of the steroid nucleus is a structural requirement for the induction of oospore formation in Phytophthora cactorum. The latter conclusion was inferred from the fact that cholesterol will induce formation of oospores while cholesterol will not. Elliott has also shown that there are other structural requirements. Of the eight sterols used to supplement P. cactorum, C-24 sterols produced maximal numbers of oospores while minimal numbers were produced with cholesterol and the C-24 methyl sterol ergosterol (2). The latter indicates a structural preference for alklyation to the C-24 stage of the sterol side chain. Elliott and coworkers (4, 6) have also shown that neither cycloartenol nor lanosterol induced oospore formation. The purpose of this paper is to extend the findings of Elliott by expanding the results to a number of other steroids and triterpenoids. The 21 steroids used in our study were chosen for their ability to reveal the extent to which steric and nuclear changes in the molecule affect sexual reproduction in P. cactorum. The triterpenoids were chosen because some pentacyclic compounds e.g., an oxidation product of β-amyrin, have hormonal activity in higher plants (8). Therefore, it was of interest to know whether they would exert similar activity in Phytophthora. Antheridiol and 29-hydroxyporiferasterol were incubated with the fungus because it was possible they might act as hormones in the Pythiaceae.

**MATERIALS AND METHODS**

**Chemicals.** Twenty one steroids were used in the present work. Cholesterol, 6-ketocholestanol, and stigmasterol were recrystallized commercial samples. H. Kircher supplied Δ^5-ergostenol, Δ^6-ergostadienol, ergostadienol, ergostanol, campesterol, and cholesterol. M. Thompson supplied sitosterol, 26-homocolesterol, 22cis-dehydrocholesterol, sitosterol, 20α-hydroxycholesterol and 20α-cholesterol (in which the conformer is skew and probably preferred) are the gifts from W. R. Nes. Spinasterol, chondrillasterol, fucosterol, saringosterol, and ergosterol were previously isolated and spectroscopically identified (18, 20, 21). All compounds except 26-homocholesterol and saringosterol have been shown to be configurationally pure samples by nuclear magnetic resonance spectroscopy (12, 15, 16, 21). Prior to incubation, each steroid was recrystallized and its GLC behavior relative to cholesterol (RRT) was determined. The GLC characteristics of many of the compounds used in the present study have previously been reported (19).

Antheridiol and 29-hydroxyporiferasterol were gifts from T. McMorris. Lupeol, betulin,oleanolic acid, and cycloartenol were gifts from H. Kircher. Cucurbitacin C was a gift from M. Thompson. Hop-22(29)-ene was a gift from A. Marsilli, and lanosterol was a commercial sample purified by Sephadex LH-20.

As previously discussed, 1% SP-1000 is a useful phase with which to separate a number of structurally similar steroids, e.g., Δ^5-sterols from Δ^6-sterols (13). Examples and relative purity of

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**ABSTRACT**

The comparative biological activity of 21 naturally occurring or synthetically derived steroids, 7 tetracyclic and pentacyclic triterpenoids, and antheridiol incubated with cultures of Phytophthora cactorum has been examined. There was greater dependence on precise steric features of the sterol side chain than on the extent of nuclear unsaturation in inducing oospore formation. There was significant effect on oospore formation by changing nuclear unsaturation in ring B from Δ^2 to Δ^7 or ωΔ^5. Converting the unsaturated sterol to its corresponding stanol resulted in a significant reduction in the number of oospores produced. The effectiveness of sterols bearing different side chains in inducing oospores was found to be in the following relative order: 24α-ethyl = trans-Δ^2,24α-ethyl > trans-Δ^2,24β-ethyl = 24α-ethylidene > 24α-methyl > 24β-methyl = trans-Δ^2,24β-ethyl > 26-methyl = saturated C; side chain and C-20 R (17αH, 20αH, right-handed conformer) = cis-Δ^4,25C; side chain and C-20 R > saturated C; side chain and C-20 S (17αH, 20βH, right-handed conformer) > no sterol = 29-hydroxyporiferasterol = 20α-hydroxycholesterol = 24α-hydroxy-24-vinylcholesterol. Of the sterols examined the most significant stereochemical criterion for the induction of oospore formation was absence of bulk on the front face of C-20. This follows from the observation that 20α-isocolesterol and 20α-hydroxycholesterol, in which a methyl and hydroxy group, respectively, project to the front in the right handed conformation, were inactive in stimulating production of oospores. None of the triterpenoids studied induced oospore formation to any significant degree. Oospore formation was not induced by antheridiol nor 29-hydroxyporiferasterol in combination or added separately to growing cultures of P. cactorum in the concentration range 0.01 – 10.0 milligrams per liter.

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4 Abbreviations: RRT, relative retention time.
various steroids incubated with *P. cactorum* using this liquid phase are given below. Cholesterol was the standard for determination of the RRT. 20-Isocolesterol, 0.93 (99%); 26-homocholesterol, 1.35 (92%); fucosterol, 1.72 (93%); campesterol, 1.19 (95%); campestanol, 1.29 (99%); ergostanol, 1.19 (99%); sitosterol, 1.44 (99%); sitostanol, 1.55 (99%). The purity of the other sterols was not less than 95%. Since not all compounds approached 100% purity, a likelihood exists that the minor components played a factor in the induction of sexual reproduction. In those cases where there were minor components, only the major component's stereochemistry was determined. The one exception was for cis Δ5-dehydrocholesterol (92% pure) which contained 8% Δ2-trans-dehydrocholesterol. We believe this is unlikely to be a factor in the induction of sexual reproduction. In those cases where the mycelial mat face down onto fresh medium in both stock transfers as well as for incubation with sterols. No attempt was made to keep the continuous culture free of light or at constant temperature. The experiment was performed twice; i.e., 2 trials were run at one temperature range and were repeated again at a higher temperature range. Trials 1 and 2 were conducted at ambient temperatures (±2 °C) under normal laboratory light conditions between November and December, whereas, trials 3 and 4 were conducted between June and August at ambient temperatures (27 ± 1 °C) in the dark.

**Bioassay for the Determination of Oospore Formation.** Twenty-five ml of liquid medium containing 2% agar (w/v) was autoclaved. Steroids and triterpenoids (10 mg/liter), as the free alcohol, solubilized in ethanol (2 ml/liter) were added as the agar began to cool. Prior to solidification, the medium containing sterol or triterpenoid was dispensed in 5-ml portions into each of 5 (50 mm) glass Petri dishes. In the case of sterol-treated cultures, there were 110 assay dishes (21 steroid treatments and control), and all dishes were inoculated on the same day as previously described. The cultures were incubated at room temperature for 21 days in the dark. Triterpenoids and antheridiol were incubated in a similar manner, except, sitosterol-treated cultures were incubated as controls.

Oospores were counted by examining 4 strip transects taken from the edge to the center of the colony similar to the method of Elliott (2). Each transect was mounted on a slide and viewed in a single plane of focus through the bottom of the slide at 100X magnification. It was not possible to count all oospores on all dishes at the time of harvest. At least one transect/dish was counted on the 21st day. Those that were not counted were placed in cold storage at +4 °C. All dishes were counted within 1 week of harvest. Cold storage had no apparent effect on the number of thick-walled resistant oospores formed. Twenty-one days was chosen as the time for harvest in order to insure that the time element was not a limiting factor in our study. A sterol and triterpenoid concentration of 10 mg/liter was used because it has been shown not to limit oospore formation at this concentration (2–4, 6).

**RESULTS AND DISCUSSION**

The sterols fell into 4 main groups based on their effectiveness in stimulating oospore production. Group 1 contains those steroid treatments that stimulated maximal oospore production and are exemplified by sitosterol-treated cultures. Group 2 contains those treatments that stimulated an intermediate number of oospores relative to the number of spores produced by sitosterol treatments. Group 3 contains those treatments that generated a minimal number of spores relative to the sitosterol treatments. Group 4 contains those treatments that did not significantly induce oospore production (Table 1). The categorization of the sterol-treatments into 4 groups was an empirical decision. When the experiment was replicated (Trials) on four separate occasions, certain steroid treatments always produced decreasing numbers of spores relative to sitosterol-treated cultures. Those steroid-treatments which induced maximal numbers of spores, relative to the other treatments and control, did not differ from each other in the number of spores produced within a trial by more than ±5% on any occasion Trials 1 through 4 (Table 1). Those treatments which induced maximal numbers of spores and did not differ from each other in the numbers of spores produced were arbitrarily classed as Group 1.

**Table 1. Effect of Sterol Structure on Oospore Production**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4 Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>24α-Ethylcholesterol</td>
<td>618</td>
<td>527</td>
<td>—</td>
<td>350 1</td>
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<tr>
<td>trans-Δ5-24α-Ethylcholesterol</td>
<td>550</td>
<td>—</td>
<td>510</td>
<td>325 1</td>
</tr>
<tr>
<td>trans-Δ5-24α-Ethylalathosterol</td>
<td>560</td>
<td>510</td>
<td>—</td>
<td>340 1</td>
</tr>
<tr>
<td>trans-Δ5-24β-Ethylalathosterol</td>
<td>375</td>
<td>310</td>
<td>210</td>
<td>— 2</td>
</tr>
<tr>
<td>24-Ethylcholesterol</td>
<td>358</td>
<td>427</td>
<td>203</td>
<td>— 2</td>
</tr>
<tr>
<td>24α-Ethylendene cholesteral</td>
<td>383</td>
<td>370</td>
<td>109</td>
<td>265 2</td>
</tr>
<tr>
<td>24α-Methylcholesterol</td>
<td>355</td>
<td>301</td>
<td>221</td>
<td>116 2</td>
</tr>
<tr>
<td>24β-Methylcholesterol</td>
<td>229</td>
<td>97</td>
<td>102</td>
<td>— 3</td>
</tr>
<tr>
<td>26-Homocholesterol</td>
<td>243</td>
<td>163</td>
<td>124</td>
<td>148 3</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>230</td>
<td>271</td>
<td>72</td>
<td>106 3</td>
</tr>
<tr>
<td>22-cis-Dehydrocholesterol</td>
<td>258</td>
<td>300</td>
<td>—</td>
<td>152 3</td>
</tr>
<tr>
<td>24α-Methylcholestanol</td>
<td>102</td>
<td>190</td>
<td>50</td>
<td>— 3</td>
</tr>
<tr>
<td>20-Isocolesterol</td>
<td>17</td>
<td>14</td>
<td>0</td>
<td>0 3–4</td>
</tr>
<tr>
<td>24α-Methylcholestanol</td>
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<td>13</td>
<td>0</td>
<td>0 4</td>
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<tr>
<td>Cholesterol</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0 4</td>
</tr>
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<td>20α-Hydroxy cholesteral</td>
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<td>0</td>
<td>4</td>
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<td>24α-Hydroxy-24-vinyl-cholesterol</td>
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<td>0</td>
<td>4</td>
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<tr>
<td>29-Hydroxycholesterol</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Control (sterol-free)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

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Those steroid treatments classed as Group 2 differed from Groups 1 and 3 in the number of spores formed by at least 10% but did not differ from each other by more than ± 5% (number of spores). Group 2 differed from Group 1 also by a factor of at least 10%, i.e., Group 2 produced 10% less spores than Group 1.

Recently Elliott and Sansome (7) have shown that oospores abort to a different extent when different sterols are added to the culture medium (7). The latter may account for the lack of reproducibility between trials for certain steroid treatments (c.f. Table 1). Further research at the molecular level is required to clarify this matter.

When P. cactorum cultures were supplemented with the homologous series of Δ^5-sterols with an H-atom, CH₃-group, and C₅H₅-group at C-24 and their corresponding stanols, greater oospore formation occurred with the Δ^5-series of sterols (Table 1). Although we did not have 24β-ethylcholestrol, (Δ^5-24α-ethylcholesterol), we did incubate the epimeric pair trans-Δ^5-24α-ethylcholestrol (spinasterol) and trans-Δ^5-24β-ethylcholestrol (choandrenisterol); the 24α-ethyl isomer resulted in production of more oospores than the 24β-isomer. The results demonstrate that in addition to recognizing changes in the nuclear condition of the sterol molecule, the fungus recognizes steric differences at C-24 in the side chain with a preference for α-ethyl sterols. Apparently, the number of oospores produced is a function of the degree of nuclear unsaturation coupled with the extent of side chain alkylation, and configuration of these additional alkyl groups. Thus, the effect of nuclear unsaturation is not an "all-or-none" phenomenon but rather a quantitative one and removal of unsaturation in ring B simply lowers activity. Measurable activity is lost only when the absence of a double bond in the nucleus is coupled with other undesirable structural features, i.e., absence of alkylation, or converting the configuration from "α" to "β" of the alkyl group at C-24.

Activity was also abolished by adding bulk on the front face of C-20. This was accomplished by adding an hydroxyl group to C-20 (20α-hydroxy cholesterl) or by inverting the stereochemistry at C-20 (20-isocholesterol) such that a methyl projects to the front in the right-handed configuration (Fig. 1). The inability of saringosterol (24α-hydroxy-24-vinylcholesterol) and 29 hydroxy poriferasterol to induce oospore formation (Table 1) may also have its origin in the increased bulk of the added hydroxyl group at C-24 and C-29 rather than at C-20. Adding a methyl group at C-24 does not lengthen the side chain, but addition of a methyl to one of the terminal isopropyl carbons of the side chain as in 26-homocolesterol does lengthen the molecule. The results show lengthening the side chain by one carbon atom has no significant effect on activity. 6-Ketocholesterol did not induce oospore formation.

*Phytophthora cactorum* was supplemented with a series of unsaturated 24β-methyl compounds, viz., Δ^5-ergostanol, Δ^5-ergostanol, Δ^5,25-ergostadienol, Δ^7,25-ergostadienol, and Δ^5,7,25-ergosta-trienol, and all 24β-methyl compounds induced the same relative number of oospores as 24β-methylcholesterol (Δ^5-ergostanol). Apparently, at the concentration of sterol added to the culture medium the introduction of various forms of unsaturation, i.e., at Δ^5, Δ^7, Δ^5, Δ^5,25, has no significant effect on sexual reproduction. This, however, was not surprising because Knights and Elliott (11) have shown that Δ^5-, Δ^7-, and Δ^5,25-steroids are converted to Δ^5-sterols. None of the triterpenoids induced oospore formation except for β-amyrin. The latter triterpenoid routinely induced 1–5% of the number of oospores formed by cholesterol treatments; this finding is anomalous.

We have incubated many of the compounds used in the present study in liquid culture to examine the effect of steroid structure on vegetative growth of *P. cactorum* and all Δ^5-sterols and cholestanol were reisolated unchanged as the free alcohol at about 0.01% free-sterol/dry weight mycelium (17). The alkylated stanols and triterpenoids have also been reisolated from the fungus (Nes, Patterson, and Bean, unpublished observations) and there was no detectable conversion of the stanol to an unsaturated sterol nor conversion of tetracycl or pentacyclic triterpenoid to a desmethyl sterol. The latter findings demonstrate that absence of activity or lowered activity was not due to a failure of the sterols or triterpenoids to enter the cells.

Side chain unsaturation at C-22(23) and C-24(28) appeared to alter activity slightly as a function of the configuration of the double bond introduced (Table 1). For instance, stigmastanol (trans-Δ^5,24α-ethylcholesterol) produced the same number of oospores as sitosterol, while 22-cis-dehydrocholesterol was slightly more active than cholesterol.

When *P. cactorum* was incubated with antheridiol, with 29 hydroxy poriferasterol (a possible intermediate to oogoniol), and with a mixture of the two, at concentrations in the range 0.01 to 10.0 mg/liter, no oospores were produced.

The results of the present investigation show *Phytophthora* possesses recognition factors that discriminate between structural changes in the sterol side chain and nucleus as well as between sterols and structurally related compounds i.e., triterpenoids. Maximal activity was not observed with sterols natural to fungi i.e., sterols bearing two H-atoms, a β-oriented methyl group, or an ethylidene group at C-24 (14), but rather with sterols natural to higher evolved tracheophytes, i.e., sterols bearing an α-oriented ethyl group (16). This suggests there may have been an evolutionary adaptation, presumably via parasitism, by *P. cactorum's* recognition factors for C-24 α-ethyl sterols. The latter sterols are known to be normally biosynthesized by the host plant (14).
In summary obligatory structural requirements of sterols for inducing sporulation were determined to be a 3β-hydroxyl function, an acyclic side chain of 8 to 10 carbon atoms having a configuration at C-20 of "R" and lacking hydroxyl groups at C-20, C-24, and C-29, and a planar tetracyclic nucleus (trans A/B ring juncture) lacking methyl moieties at C-4, and C-14. For oospore production, an interplay also existed between the configuration and the extent of alkylation at C-24 of the sterol molecule. Thus, α-ethyl sterols promoted maximal oospore production while β-methyl sterols promoted minimal oospore production.

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