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Effect of Steric and Nuclear Changes in Steroids and Triterpenoids on Sexual Reproduction in *Phytophthora cactorum*[1]

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ABSTRACT

The comparative biological activity of 21 naturally occurring or synthetically derived steroids, 7 tetracyclic and pentacylic 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 no significant effect on oospore formation by changing nuclear unsaturation in ring B from Δ^5 to Δ^7 or to $\Delta^{5,7}$. 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^{22}-24α-ethyl > *trans*-Δ^{22}-24β-ethyl = 24α-E-ethylidene = 24α-methyl > 24β-methyl = *trans*-Δ^{22}-24β-methyl = 26-methyl = saturated C_7 side chain and C-20 R (17-αH, 20-αH, right-handed conformer) = *cis*-Δ^{22}-C_7 side chain and C-20 R > saturated C_7 side chain and C-20 S (17-αH, 20-βH, right-handed conformer) > no sterol = 29-hydroxyporiferasterol = 20α-hydroxycholesterol = 24ξ-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 20-isocholesterol 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.

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 cholestanol will not. Elliott has also shown that there are other structural requirements. Of the eight sterols used to supplement *P. cactorum*, C-24 ethyl 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 alkylation to the C-2 stage at C-24 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, $\Delta^{5,22}$-ergostadienol, ergostanol, campestanol, campesterol, and cholestanol. M. Thompson supplied sitostanol, 26-homocholesterol, 22 *cis*-dehydrocholesterol. Sitosterol, 20αhydroxycholesterol and 20-isocholesterol (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[4]) 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^0-sterols (13). Examples and relative purity of

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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-Isocholesterol, 0.93 (>99%); 26-homocholesterol, 1.35 (>92%); fucosterol, 1.72 (>93%); campestanol, 1.19 (>95%); campesterol, 1.29 (>99%); ergostanol, 1.19 (>99%); sitostanol, 1.44 (>99%); sitosterol, 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* Δ^{22}-dehydrocholesterol (92% pure) which contained 8% Δ^{22}-*trans*-dehydrocholesterol. We believe it is unlikely that the minor components affected the induction of reproduction because the concentration of the sterol needed to induce maximal oospores approaches 10 mg/liter. Since we added our sterol at 100 mg/liter, the concentration of minor components would probably be limiting and therefore biologically inconsequential in its effect on reproduction.

All triterpenoids used in the study were greater than 95% pure (except betulin which was 60% pure) as determined by GLC using a liquid phase of 1% SE-30. Some examples of RRT using this phase and not previously reported in the literature are as follows: lupeol, 1.89; hop-22(29)-ene, 1.39; β-amyrin, 1.59.

Phytophthora cactorum, culture—strain IMI 21168, was the gift of C. Elliott. The fungus was grown at room temperature (25 ± 5 C), on a synthetic sterol-free sucrose-asparagine medium described by Elliott (2). Added sterol was solubilized in ethanol, Tween 80 was not added to the medium, and 20 g/liter of agar was added to solidify the medium (17). Agar-supplemented medium is operationally defined as "solid medium". The stock culture was maintained by transferring 5-mm plugs onto fresh solid medium every 2 weeks. We routinely placed the plug with 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 (21 ± 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 alcohols, solubilized in ethanol (2 ml/liter) were added as the agar began to cool. Prior to solidification, the medium containing steroid 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 100× magnification. It was not possible to count all transects 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 stimulated 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 I. *Effect of Sterol Structure on Oospore Production*

Each value under Trial number represents the mean oospore count of 4 radial transects in each of 5 Petri dishes within a trial. The coefficient of variation of total counts per 5 Petri dishes representing one sterol treatment within a trial rarely exceeded 10%. Confidence limits for most mean values based on the t-distribution are at the 95% (confidence) level. Dash lines represent contamination. Trials 1 and 2 were grown under normal laboratory light conditions at ambient temperatures (22 ± 2C), trials 3 and 4 in the dark at 27 ± 1C. Groups represent the categorizations of sterol-treatments into their effectiveness in stimulating oospore production. The segregation of the sterol treatments into 4 groups is based on a 10% or greater difference in the mean number of oospores produced per treatment.

Treatment	Trial Number[a]				
	1	2	3	4	Groups
24α-Ethylcholesterol	618	527	—	350	1
trans-Δ^{22}-24α-Ethylcholesterol	550	—	510	325	1
trans-Δ^{22}-24α-Ethyllathosterol	560	510	—	340	1
trans-Δ^{22}-24β-Ethyllathosterol	375	310	210	—	2
24E-Ethylidene cholesterol	358	427	203	—	2
24α-Ethylcholestanol	383	370	109	265	2
24α-Methylcholesterol	355	301	221	116	2
24β-Methylcholesterol	229	97	102	—	3
26-Homocholesterol	243	163	124	148	3
Cholesterol	230	271	72	106	3
22-*cis*-Dehydrocholesterol	258	300	—	152	3
24α-Methylcholestanol	102	190	50	—	3
20-Isocholesterol	17	14	0	0	3–4
24β-Methylcholestanol	11	13	0	0	4
Cholestanol	3	2	0	0	4
20α-Hydroxy cholesterol	0	0	0	—	4
24ξ-Hydroxy-24-vinyl-cholesterol	0	0	—	0	4
29-Hydroxycholesterol	0	0	—	0	4
Control (sterol-free)	0	0	0	0	4

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.

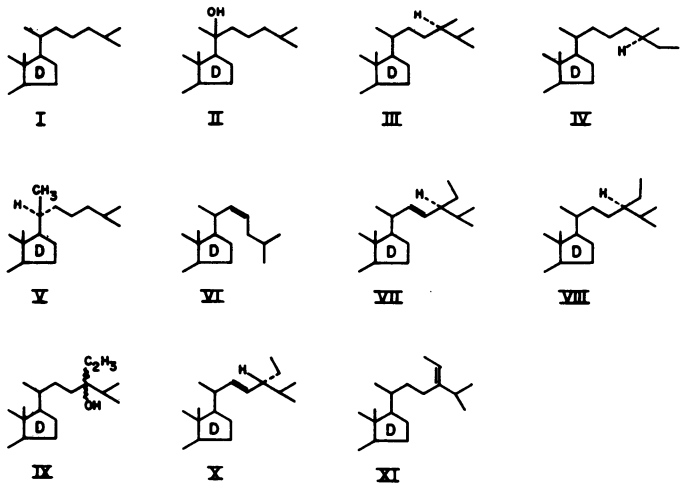
Recently Elliott and Sansome (7) have shown that oospores abort to a different extent when different sterols are added to the culture medium (7). The latter may account for the lack of reproducibility between trials for certain sterol treatments (c.f. Table 1). Further research at the molecular level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

When *P. cactorum* cultures were supplemented with the homologous series of Δ^5-sterols with an H-atom, CH_3-group, and C_2H_5-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-ethyllathosterol, (Δ^7-24β-ethylcholesterol), we did incubate the epimeric pair trans-Δ^{22}-24α-ethyllathosterol (spinasterol) and trans-Δ^{22}-24β-ethyllathosterol (chondrillasterol); 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 cholesterol) or by inverting the sterochemistry at C-20 (20-isocholesterol) such that a methyl projects to the front in the right-handed conformation (Fig. 1). The inability of saringosterol (24ξ-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-homocholesterol does lengthen the molecule. The results show lengthening the side chain by one carbon atom has no significant effect on activity. 6-Ketocholestanol did not induce oospore formation.

Phytophthora cactorum was supplemented with a series of unsaturated 24β-methyl compounds, viz., Δ^5-ergostenol, Δ^7-ergostenol, $\Delta^{5,22}$-ergostadienol, $\Delta^{7,22}$-ergostadienol, and $\Delta^{5,7,22}$-ergostatrienol, and all 24β-methyl compounds induced the same relative number of oospores as 24β-methylcholesterol (Δ^5-ergostenol). Apparently, at the concentration of sterol added to the culture medium the introduction of various forms of unsaturation, *i.e.*, at Δ^5, $\Delta^{5,7}$, Δ^7, $\Delta^{5,22}$ has no significant effect on sexual reproduction. This, however, was not surprising because Knights and Elliott (11) have shown that $\Delta^{5,7}$- and Δ^7-sterols 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 sterol 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-



Number	Trivial name	Structure based on changes or additions to 5α-cholestan-3β-ol
I	Cholesterol	Δ^5
II	20α-Hydroxycholesterol	Δ^5-20α-OH
III	Campesterol	Δ^5-24α-methyl
IV	26-Homocholesterol	Δ^5-26α-methyl
V	20-Isocholesterol	Δ^5, 20*S*
VI	22-*cis*-Dehydrocholesterol	Δ^5, *cis*-Δ^{22}
VII	Stigmasterol	Δ^5, *trans*-Δ^{22}-24α-ethyl
VIII	Sitosterol	Δ^5, 24α-ethyl
IX	Saringosterol	Δ^5, 24ξ-hydroxy-24-vinyl
X	Chondrillasterol	Δ^7, *trans*-Δ^{22}-24β-ethyl
XI	Fucosterol	Δ^5, *trans*-24-ethylidene

[a] The configuration at C-20 is "R" except for number V which is "S".

FIG. 1. Sterol side chain structures of many of the compounds incubated in the present study.[a]

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 tetracyclic 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, stigmasterol (trans-Δ^{22}-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, α 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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