Repellent Activity of Estrogenic Compounds toward Zoospores of the Phytopathogenic Fungus *Aphanomyces cochlioides*

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Screening chemical compounds, we found that a xenoestrogen, bisphenol A, showed potent repellent activity against the zoospores of *Aphanomyces cochlioides*. Based on this finding, we tested a number of androgenic and estrogenic compounds (e.g. testosterone, progesterone, estradiols, diethylstilbestrol, estrone, estriol, pregnenolone, dienestrol etc.) on the motility behavior of *A. cochlioides* zoospores. Interestingly, most of the estrogenic compounds exhibited potent repellent activity (1 μg/ml or less by the "particle method") toward the motile zoospores of *A. cochlioides*. We derivatized some of the estrogens and discussed the relationship between the structure of active molecules and their repellent activity. Apparently, aromatization of the A ring with a free hydroxyl group at C-3 position of a steroidal structure is necessary for higher repellent activity. Interestingly, methylation of diethylstilbestrol (DES) yielded completely different activity i.e. both mono- and di-methyl ethers of DES showed attractant activity. Moreover, the attracted zoospores were encysted and then germinated in the presence of di-methyl ether of DES. The potential usefulness of this repellent test is discussed for the detection of estrogenic activity of naturally occurring compounds, and the possible role of phytoestrogens in host/parasite interactions. So far, this will be the first report of repellent activity of estrogenic compounds toward trivial fungal zoospores.

Introduction

Aphanomyces cochlioides Drechsler (Saprolegniaceae; Oomycetes) causes damping-off and root rot diseases of sugar beet and spinach (Drechsler, 1928; Ui and Nakamura, 1963). It also infects several other species of Chenopodiaceae and Amaranthaceae (Ui and Nakamura, 1963). Zoospores of *A. cochlioides* are attracted to the host roots by chemotaxis where they aggregate as a hemispheric mass on the surface of roots, encyst, germinate and penetrate into the root tissues. These phenomena are believed to be regulated by host-mediated chemical signals (Rai and Strobel, 1966; Yokosawa *et al.,* 1988; Horio *et al.,* 1992, 1993). We identified cochliophilin A (**1**), as a host-specific attractant of the zoospores of *A. cochlioides* from the roots of spinach (Horio *et al.*, 1992). Compound **1** has also been reported in sugar beet (Takahashi *et al.*, 1987). A phenolic amide (*N-trans*-feruloyl-4-*O*-methyldopamine, **2**) was also isolated from the roots of another host plant *Chenopodium album*, and identified as a potent attractant of *A. cochlioides* zoospores (Horio *et al.,* 1993).

We hypothesized that the roots of non-host plants may exude chemical signals responsible for their resistance. Based on this hypothesis, some bioactive compounds were isolated from non-host plant sources like zoospores motility inhibiting factors from *Portulaca oleracea* (Mizutani *et al.,* 1998), lytic factors from *Ginkgo biloba* (Tahara *et al.,* 1999), repellent factors from *Dalbergia odorifera* (Islam and Tahara, 2001). In an extensive screening of non-host plant extracts, we found that the viability and motility behavior of *A. cochlioides* zoospores were markedly regulated in the presence of plant extracts. Interestingly, the zoospores of *A. cochlioides* were found to be almost unaffected by reputed cytotoxins like, vincristine, vinblastine, bleomycin hydrochloride, aphidicolin,

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paclitaxel, forskolin, (3a*R*)-(+)-sclareolide, (*S*)-(+)-camptothecin *etc.* at about 1000 μg/ml (particle method) (Islam and Tahara, 2001).

In a parallel screening we found that bisphenol A (BPA) (**3**), a reputed xenoestrogen (Takai *et al.,* 2000), exhibited potent repellent activity against the zoospores of *A. cochlioides.* BPA (**3**) is a constituent of polycarbonate plastics, epoxy and polystyrene resins that are used intensively in the food-packing industry and in dentistry. In several experiments, effects of BPA (**3**) on mammalian systems resemble those of 17β-estradiol (Krishnan *et al.*, 1993; Steinmetz *et al.,* 1998). The actions of **3** were found to be mediated through the estrogen receptor, a ligand-dependent transcription factor that regulates estrogen-responsive genes (Hiroi *et al.,* 1999; Takai *et al.,* 2000).

Occurrence of mammalian sex hormones in plants have been often reported (Heftmann *et al.,* 1966; Heftmann, 1975). Their sporadic availability and the chemical role in plants were discussed by Heftmann (1975) and Harborne (1993). The fact that human sex hormones, both male and female, occur in trace amounts in a number of plants is now well founded, but there is as yet no explanation for these occurrence (Harborne, 1993). It is difficult to screen plants for their occurrence due to their low level in plants. This prompted us to study the effects of mammalian sex hormones, and their derivatives on the motile zoospores of phytopathogenic *A. cochlioides.*

Materials and Methods

General

The silica gel 60 ASTM mesh 230–400 was used for column chromatography while purity of the samples was checked on Merck Kieselgel 60 F_{254}, 0.2 mm thick TLC plates. The spots were viewed under 254 and 365 nm UV light and spraying with 5% H_2SO_4 in EtOH. The mass spectra were recorded on a JEOL JMS-SX102A (FD) mass spectrometer and a JEOL JNM-EX 270 for recording the ^1H NMR. TMS was used as the internal standard in NMR spectrometry.

Materials

All chemicals commercially available were of the highest purity and unless otherwise stated were used without further purification. Bisphenol A (**3**), diethylstilbestrol (DES) (**4**), estrone (**5**), 17α-estradiol (**6**), 17β-estradiol (**7**), estriol (**8**), dienestrol (**9**), testosterone (**10**), pregnenolone (**11**), progesterone (**12**) and 3-*O*-benzoyl-17β-estradiol (**23**) were purchased from reputed pharmaceutical companies. Formononetin was isolated from *D. odorifera* (Islam and Tahara, 2001), and miroestrol was (a gift) from Dr.. J. L. Ingham, University of Reading, UK. One metabolite of testosterone (TES-1, **20**), and two metabolites of pregnenolone (PRE-4, **21**; PRE-5, **22**) were obtained from the biotransformation of **10** and **11**, respectively by *Botrytis cinerea* (Farooq and Tahara, 2000). Cochliophilin A (**1**) and *N-trans*-feruloyl-4-*O*-methyldopamine (**2**) used in this experiment were synthesized by Horio *et al.* (1992, 1993).

Production of zoospores and bioassay

The fungus *A. cochlioides* (AC-5) was cultured for 3–4 days on a corn meal agar (Difco) plate at 20 °C. The production of zoospores and the "particle bioassay" were carried out as previously reported (Horio *et al.,* 1992; Mizutani *et al.,* 1998). Briefly, one drop of solution of each chemical dissolved in EtOAc or acetone, and adjusted to an appropriate concentration, was dropped onto a few particles of Chromosorb W AW (60–80 mesh) on a watch glass. Excess solution was immediately absorbed by a tip of filter paper and the particles were allowed to evaporate the solvent. One to two of these particle(s) were carefully dropped into 2 ml of a zoospore suspension (10^5/ml) in a small Petri dish (3 cm i.d.), and the motility behavior of the zoospores around the particle(s) was observed microscopically up to 10–15 min after addition of the particle(s). Control particles were treated with solvent alone. Around particles treated with an inactive compound, the zoospores moved in an unvarying, regular manner and at a constant speed. In contrast, zoospores close to particle(s) treated with any active compounds responded in one of the following ways. 1) Attractant activity: relatively large number of zoospores assembled around the particle(s), moving with increased speed in a complex zigzag or circular manner. There was a clear gradient in zoospore density which decreased with increasing distance from the particle. 2) Repellent activity: zoospores repelled

from the treated particle(s) and not approaching to the particle(s) quickly became surrounded by a circular, zoospore-free zone. 3) Stimulant activity: zoospore movement near the particles increased in speed without any variation in zoospore density. 4) Encystment activity: zoospores stopped their motility, and changed into spherical spores sorrounded by the cell wall called cystospores. The cystospore is a thick-walled and non-motile spore that is relatively resistant to environmental stresses and can germinate when specific signal substance(s) are present.

Derivatization of DES and 17β-estradiol

Commercially pure DES (**4**) and 17β-estradiol (**7**) (each 100 mg) were acetylated at room temperature for 12 h with equal volume of pyridine and acetic anhydride. After the acetylation process, the reaction mixture was purified by preparative thin-layer chromatography (PTLC) in *n*-hexane – EtOAc = 3:1 v/v to give di-acetates of **4** and **7**. Mono- and di-methylation of DES, and monomethylation of 17β-estradiol were done using suitable ratio of dimethyl sulfate in a mixture of K_2CO_3 and acetone, and the purification was done using similar technique applied to the acetates.

The aromatic hydroxyl group of 17β-estradiol was selectively acetylated by reacting with an equal mol of KOH in acetone followed by the addition of 1.2 mol of acetic anhydride at room temperature for 6 h. 3-*O*-Acetyl-17β-estradiol (**24**) was purified by PTLC using the above solvent system. 17-*O*-Acetyl-17β-estradiol (**25**) was prepared by the hydrolysis of 3,17-*O*-diacetyl-17β-estradiol using 10 equivalents of *n*-butylamine (*n*-$BuNH_2$) in benzene at room temperature for 24 h (Bell, 1986) and purified by silica gel CC using *n*-hexane – EtOAc – MeOH = 3:1:0.3 v/v. The identity of all derivatives was confirmed by mass spectroscopy and 1D ^{1}H NMR.

Results

Effects of estrogenic compounds and their derivatives

We purchased some reputed commercial mammalian sex hormonal substances, for example, bisphenol A (**3**), diethylstilbestrol (DES) (**4**), estrone (**5**), 17α-estradiol (**6**), 17β-estradiol (**7**), estriol (**8**), dienestrol (**9**), testosterone (**10**), pregnenolone (**11**), and progesterone (**12**). Also two natural estrogenic mimics, formononetin (**13**) and miroestrol (**14**) (Jones and Pope, 1960) were included into the zoospores bioassay. After a preliminary bioassay using particle method, we derivatized two representative estrogenic compounds, DES (**4**) and 17β-estradiol (**7**) to their methylated and acetylated derivatives (compounds **15–19**) to establish the relationship between structure and repellent activity. In the bioassay, we also added one metabolite of testosterone (TES-1, **20**) and two metabolites of pregnenolone (PRE-4, **21**; PRE-5, **22**, Farooq and Tahara, 2000), and a synthetic 3-*O*-benzoyl-17β-estradiol (**23**). Two acetyl derivatives of 17β-estradiol, compounds **24** and **25** were also prepared and tested.

The bioassay results of mammalian sex hormonal substances and their derivatives toward the zoospores of *A. cochlioides* zoospores are presented in Table I. Most of the hormonal substances (compounds **3-11**) exhibited repellent activities against the motility of the zoospores except progesterone (**12**) and the natural mimic miroestrol (**14**). The highest activity was recorded in DES (**4**), 17β-estradiol (**7**) and estriol (**8**) (active at 0.5 μg/ml), followed by 17α-estradiol (**6**), estrone (**5**), dienestrol (**9**) (active at 1.0 μg/ml), testosterone (**10**) (active at 50 μg/ml) and pregnenolone (**11**) (active at 100 μg/ml). The natural mimic, miroestrol (**14**) did not show any activity up to 1000 μg/ml whereas another estrogenic natural product, formononetin (**13**) showed attractant/stimulant activity towards *A. cochlioides* zoospores at 50 μg/ml. It revealed that most of the active estrogenic compounds except pregnenolone (**11**) showed higher repellent activity than that of an androgen, testosterone (**10**) The xenoestrogen, bisphenol A (**3**) showed clear repellent activity at 1 μg/ml under the same bioassay condition.

The acetylated and methylated products (**15–17, 24** and **25**) of DES (**4**) and 17β-estradiol (**7**), showed relatively lower but the same kind of bioactivity as the mother compounds (Table I) except the methylated products of DES (**18** and **19**). Interesting to note that methylated DES (**18** and **19**) showed a completely different bioactivity towards the zoospores. DES dimethyl ether (**19**), displayed potent attracting activity followed by encystation of zoospores within 4–5 min at 10 μg/ml concen-

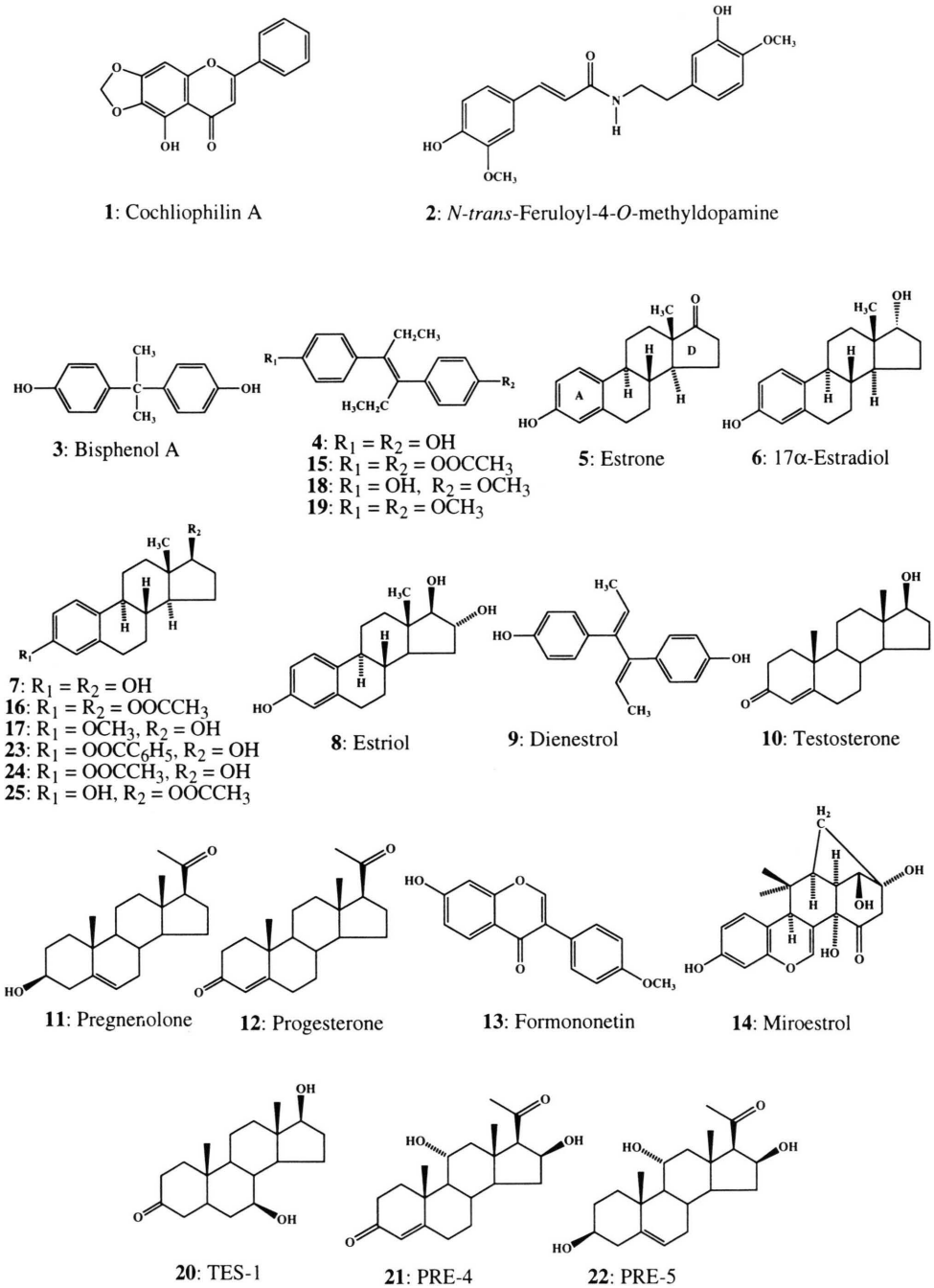

Fig. 1. Structures of two host-specific attractants, and some mammalian sex hormonal substances and their derivatives tested for the motility behavior of zoospores of the phytopathogenic fungus *Aphanomyces cochlioides*.

tration whereas DES (**4**) is a potent repellent. The encysted zoospores were found to germinate at the bottom of petri dish after 40–60 min of treatment. However, lower concentration (1.0 μg/ml) of **19** just attracted and stimulated zoospores without ceasing the motility of attracted zoospores. It was

Table I. Bioactivity of some mammalian sex hormonal substances and their derivatives toward *Aphanomyces cochlioides* zoospores.

Name of estrogenic compounds and their derivatives	Observed activity of estrogenic compounds and their derivatives [μg/ml]*							
	1000	100	50	10	5	1	0.5	0.1
Diethylstilbestrol (DES) (**4**)	nt	---	---	--	--	-	-	±
17α-Estradiol (**6**)	nt	---	---	--	--	-	±	na
17β-Estradiol (**7**)	nt	---	---	--	--	-	-	±
Estrone (**5**)	nt	---	---	--	--	-	±	na
Estriol (**8**)	nt	---	---	--	--	-	-	±
Dienestrol (**9**)	nt	---	---	--	-	-	±	na
Bisphenol A (**3**)	---	---	--	--	-	-	na	nt
Testosterone (**10**)	---	--	-	na	nt	nt	nt	nt
Pregnenolone (**11**)	--	-	±	na	nt	nt	nt	nt
Estradiol benzoate (**23**)	na	na	nt	nt	nt	nt	nt	nt
Progesterone (**12**)	na	na	nt	nt	nt	nt	nt	nt
PRE-4 (**21**)**	na	na	nt	nt	nt	nt	nt	nt
PRE-5 (**22**)**	na	na	nt	nt	nt	nt	nt	nt
TES-1(**20**)***	na	na	nt	nt	nt	nt	nt	nt
Miroestrol (**14**)	na	na	nt	nt	nt	nt	nt	nt
Formononetin (**13**)	+++/sss	++/ss	+/s	na	na	nt	nt	nt
DES diacetate (**15**)	---	---	-	±	na	na	nt	nt
Estradiol diacetate (**16**)	---	--	-	-	±	na	na	nt
3-*O*-Methyl estradiol (**17**)	---	--	-	±	na	na	nt	nt
DES Dimethyl ether (**19**)	+++&e	+++&e	+++&e	++&e	+&s	+&s	±	na
DES Monomethyl ether (**18**)	+++/sss	+++/sss	++/ss	+/s	s	±	na	na
Estradiol 3-acetate (**24**)	---	---	---	--	-	±	na	na
Estradiol 17-acetate (**25**)	---	---	---	--	-	-	±	na

Note: * Particle method; na = non-active; nt = not tested; – = repellent, + = attractant; e = encysting; s = stimulant; ± = activity is not clear well.
** PRE-4 (**21**) and PRE-5 (**22**) are two metabolites of **11** (Farooq and Tahara, 2000).
*** TES-1 (**20**) is a metabolite of **10** (Farooq and Tahara, 2000).

observed that the attracted zoospores seemed to search something by their flagella on the surface of treated particles. Before releasing their flagella at 10 μg/ml, the zoospores also wound up their flagella around their body or moved in a circular fashion. On the other hand, DES monomethyl ether (**18**) showed attracting and stimulating activity at 10 μg/ml without impairing the motility of zoospores. Mizutani *et al.* (1998) observed that zoospores of *A. cochlioides* were halted by the action of two different compounds, while individually applied one compound (1-linoleoyl-2-lysophosphatidic acid monomethyl ester) had repellent and the other (*N-trans*-feruloyltyramine) exhibited stimulant activity. The microbial metabolites of testosterone (TES-1, **20**) and pregnenolone (PRE-4, **21**; PRE-5, **22**), and 3-*O*-benzoyl-17β-estradiol (**23**) did not show any activity toward the zoospores of *A. cochlioides* at 1000 μg/ml.

Effects of estrogens in co-existence with the host-specific attractant, cochliophilin A

The effects of potent repellent factor DES (**4**) in co-existence with host-specific attractant cochliophilin A (**1**) toward *A. cochlioides* zoospores are presented in Table II. Compound **1** alone at lower concentrations (0.0005–0.001 μg/ml) showed attractant activity. However, at higher concentrations (0.01–1.0 μg/ml) it caused encystation followed by germination of the attracted zoospores. On the other hand, DES (**4**) exhibits only repellent activity in a dose dependent – manner in a range of 1.0–100 μg/ml. Application of 1.0 μg/ml of **4** with 0.0005 μg/ml of **1** completely inactivated each other (Table II). However, concomitant application of 0.001 μg/ml of cochliophilin A (**1**) with 1.0 μg/ml of DES (**4**) caused weaker attractant and prominent stimulant responses by the zoospores. Clearly, the repellent activity of DES (4) was fully

Table II. Bioactivity of DES in co-existence with a host-specific attractant cochliophilin A toward *Aphanomyces cochlioides* zoospores.

Tested compounds [μg/ml]		Observed bioactivity of DES in co-existence with cochliophilin A				
Diethylstilbestrol	Cochliophilin A*	Attractant	Repellent	Stimulant	Encystment	Germination
1.0	0.0005	±	-	-	-	-
1.0	0.001	++	-	+	-	-
1.0	0.01	+++	-	+	++	+
1.0	0.1	+++	-	-	+++	+++
10.0	0.0005	-	+	+	-	-
10.0	0.001	++	-	+	-	-
10.0	0.01	+++	-	-	++	+
10.0	0.1	+++	-	-	+++	+++
50.0	0.001	-	+	+	-	-
50.0	0.01	+	-	+	-	-
50.0	0.1	+++	-	-	++	++
100.0	0.001	-	++	+	-	-
100.0	0.01	-	+	+	-	-
100.0	0.1	**++pb**	-	+	+	+
100.0	1.0	**+++pb**	-	-	+++	+++

* Cochliophilin A (**1**) showed clear attractant activity at 0.0005 μg/ml in the tested condition; β-estradiol (**7**) also showed similar trend of bioactivity in co-existence of cochliophilin A (data not shown); **pb** = zoospores pushed back from the particle upto 5–6 min and then attracted in usual manner. The frequency of '**+**' sign indicates the strength of bioactivity, and '**-**' sign indicates no activity under specific treatment.
Bioassay: Two treated particles (separately by two compounds) were dropped simultaneously on the petri dish containing 2 ml of zoospore suspension (particles quickly come very close to each other) and the activity of the particles toward the behavior of zoospores were observed by microscope for about 15 min for each treatment. The results presented here were confirmed by at least three times repetition under equivalent conditions.

suppressed in this combination but the attractant activity of cochliophilin A (**1**) was also weakened and somewhat modified due to the presence of **4**. Gradual increase of the concentration of **1** in combination with a constant concentration of **4** (1.0 μg/ml) displayed attractant, encystment and germination activity, and complete suppression of the activity due to **4**. On the other hand, concomitant application of higher doses of **4** and lower doses of **1** (10.0 μg/ml DES + 0.0005 μg/ml **1** or 50.0 μg/ml DES + 0.001 μg/ml **1** or 100.0 μg/ml DES + 0.01 μg/ml **1**) exhibited clear repellent and stimulant activity towards the zoospores (Table II). The quality of zoospore responses was altered due to the mixing ratios of opposite active principles (attractant and repellent). Interestingly, the attracted zoospores were pushed back instantly from the particles coated with higher amounts of both compounds (100.0 μg/ml **4** and 0.1 μg/ml **1**), and this phenomenon continued up to 5–6 min. Finally, the attractant and encystment activity of cochliophilin A (**1**) emerged but some of the rejected zoospores were encysted within 4–6 min. All encysted zoospores germinated after 40–60 min. Apparently, the receptor for estrogenic repellents was not affected directly by attractants (**1** and **2**), because the repellent activity of estrogens was observed in the zoospores suspended in the homogenous solution of **1** or **2**.

Discussion

These results show that the steroidal compounds with an aromatized A ring possess higher bioactivity toward *A. cochlioides* zoospores except 3-*O*-benzoyl-17β-estradiol (**23**). The acetylation and methylation of 17β-estradiol (**16**, **17, 24,** and **25**) and acetylation of DES (**15**) seemed to slightly decrease the repellent activity. Interestingly, however, mono- and di-methylation products of DES (**18** and **19**) exhibited a completely opposite biological activity (Table I). In case of the benzoate group at C-3-OH in 17β-estradiol (**7**), a zero activity was observed at 1000 μg/ml whereas acetylation at the same position showed a little lower repellent activity than the mother compound (**7**). Acetylation of the hydroxyl group at C-17 (**25**) did not affect much the repellent activity indicating that

the free hydroxyl group at C-3 is more significant than that on C-17. Testosterone (**10**) and pregnenolone (**11**) showed lower activity than the estrogenic compounds having aromatization in the A ring of their steroidal skeleton (**5–9**). With respect to the structure-activity relationship, it appears that aromatization of A ring together with a free hydroxyl group at C-3 position is necessary for higher repellent activity of estrogenic compounds. Other substituents at the same position (C-3) decreased the bioactivity which is related to the size of the substituents. There may be a correlation between estrogenic activity and repellent activity of steroids. In animals, the sex hormones are formed from progesterone by successive oxidation steps, both at C-17 and at C-19. The oxidative removal of the side chain leads to the C-19 series with androgenic activity, and the oxidative removal of the angular methyl group at C-10 and aromatization of A ring leads to the C-18 series with estrogenic activity (Heftmann, 1970). The powerful synthetic estrogenic compound, DES (**4**) displayed potency an equivalent to 17β-estradiol (**7**) and estriol (**8**) (all active at 0.5 μg/ml). On the other hand, a xenoestrogen, bisphenol A (**3**) exhibited repellent activity at 1 μg/ml. It has been reported that in the mammalian system, compound **3** acts through the estrogen receptor (Takai *et al.,* 2000).

Derivatives (**18** and **19**) resulting from methylation of DES (**4**) showed completely different active principles from that of the mother compound. Both mono- and di-methylation products exhibited attractant and stimulant activity toward the zoospores of *A. cochlioides*. Moreover, the activity of the di-methylated product (**19**) resembled the attractive activity of cochliophilin A (**1**) or *N-trans*-feruloyal-4-*O*-methyldopamine (**2**) isolated from the host plants of *A. cochlioides*. Compound **1** and **2**, at around a 10^{-7} and 10^{-6} M concentration, respectively, strongly attract zoospores to the treated particles and subsequently cause encystation and germination of the attracted spores within 40–60 min. Compound **19** may act through a specific receptor because it was found to be active when compound **1** or **2**, or other estrogenic compounds were suspended in the homogenous solution of motile zoospores.

The major mammalian sex hormones (both androgens and estrogens), like estrone (**5**), 17β-estradiol (**7**), estriol (**8**), testosterone (**10**) have been isolated from several higher plants (Heftmann, 1975; Harborne, 1993). Basically, steroid hormones are a group of substances derived from cholesterol which exert a very wide range of effects on biological processes such as growth, metabolism and sexual differentiation (King and Mawaring, 1974). However, our current knowledge on their effect in non-mammalian biological systems like microorganisms is limited. Both growth-inhibiting and growth promoting effects of steroidal hormones have been observed by Fitzgerald and Yotis (1971), but more interestingly, testosterone and estradiol have been found to have sex hormone activity on yeast (Takao *et al.,* 1970). An insect-repellent steroid was isolated from the Peruvian weed *Nicandra physalodes* (Bates and Eckert, 1972). Antheridiol has been identified as the chemotactic hormone of the water mold, *Achlya bisexualis* (Saprolegniaceae, Barksdale, 1969). Heftmann *et al.* (1960) observed that a structurally related sterol of anthridiol, 5α-stigmast-22-en-3β-ol, which is produced by *Dictyostelium discoideum*, triggers the remarkable differentiation which this slime mold undergoes.

In the present study, the estrogenic and repellent activities of known estrogenic compounds revealed to be correlated. The particle bioassay method is very simple and convenient for testing the motility behavior of fungal zoospores. Thus, the present repulsion test seems to be useful for pre-screening the detection of estrogenic activity of naturally occurring compounds. Therefore, it may be important to carry out further work to evaluate the usefulness of this repulsion test for the bioassay-guided isolation of similar structures of phytoestrogenic compounds. Furthermore, high repellent activity of mammalian sex hormones towards fungal zoospores may be biologically significant because such high negative chemotaxis was not yet reported for any zoosporic fungi. It may be important for biorational control of zoosporic fungi and/or in studying the molecular basis of chemoresponses of zoosporic fungi.

Phytoestrogen can sometimes be involved in plant growth and development even in the sexual expression in plants (Heftmann, 1975) but, to our knowledge, the negative chemotaxis toward fungal zoospores recorded herein has not been formerly described. Chemotaxis of fungal zoospores has been reported in many papers (Zentmyer, 1961;

Cameron and Carlile, 1978; Yokosawa and Kuninaga, 1979; Yokosawa *et al.*, 1986; Morris and Ward, 1992; Horio *et al.*, 1992; Deacon and Donaldson, 1993) but, reports on negative chemotaxis are very few (Mizutani *et al.*, 1998, Islam and Tahara, 2001). Previously, we found that monoacylated phosphatidic acid derivatives containing at least one hydroxyl group at the phosphoryl unit showed repellent activity against *A. cochlioides* zoospores (Mizitani *et al.*, 1998). (±)-Medicarpin, an isoflavonoid isolated from *Dalbergia odorifera* also exhibited repellent activity against the zoospores of *A. cochlioides* (Islam and Tahara, 2001). The mechanism of high repellent activity by mammalian sex hormonal substances on fungal zoospores is difficult to explain by our current knowledge. Negative chemotaxis by mammalian sex hormonal substances most of which are also reported in plants, raises questions on the occurrence of this phenomenon particularly during host/parasite interactions, and the speculation that minor constituents of phytoestrogens may contribute to defend non-host plants against pathogens.

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Barksdale A. W. (1969), Sexual hormones of *Achlya* and other fungi. Science **166**, 831–837

Bates R. B. and Eckert D. J. (1972), Nicandrenone, an insecticidal plant steroid derivative with ring D aromatic. J. Am. Chem. Soc. **94**, 8258–8260.

Bell K. H. (1986), Facile selective aminolysis of phenolic benzoates with 1-butanamine in benzene. Tetrahedron Lett. **27**, 2263–2264.

Cameron J. N. and Carlile M. J. (1978), Fatty acids, aldehydes and alcohols as attractants for zoospores of *Phytophthora palmivora*. Nature **271**, 448.

Deacon J. W. and Donaldson S. P. (1993), Molecular recognition in the homing responses of zoosporic fungi, with special reference to *Pythium* and *Phytophthora*. Mycol. Res. **97**, 1153–1171.

Drechsler C. (1928), The occurrence of *Aphanomyces cochlioides* n. sp. on sugar beets in the United States. Phytopathology **18**, 149.

Farooq A. and Tahara S. (2000), Biotransformation of testosterone and pregnenolone catalyzed by the fungus *Botrytis cinerea*. J. Nat. Prod. **63,** 489–491.

Fitzgerald T. and Yotis W. W. (1971), Interference with cellular incorporation of substrates into *Staphylococcus aureus* by hormones. J. Med. Microbiol. **4**, 97–106.

Harborne J. B. (1993), *Introduction to Ecological Biochemistry,* 4th edn. Academic Press, London, pp. 104–125.

Heftmann E., Wright B. E. and Liddle G. U. (1960), The isolation of Δ^{22}-stigmasten-3β-ol *Dictyostelium discoideum*. Arch. Biochem. Biophys. **91**, 266–270.

Heftmann E. (1970), Steroid Biochemistry. Academic Press, New York.

Heftmann E. (1975), Functions of steroids in plants. Phytochemistry **14**, 891–902.

Heftmann E., Ko S. T. and Bennett R. D. (1966), Identification of estrone in pomegranate seeds. Phytochemistry **5**, 1337–1339.

Hiroi H., Tsutsumi O., Momoeda M., Takai Y., Osuga Y. and Taketani Y. (1999), Differential interactions of bisphenol A and 17beta-estradiol with estrogen receptor alpha (Eralpha) and Erbeta. Endocrine Journal **46**, 773–778.

Horio T., Kawabata Y., Takayama T., Tahara S., Kawabata J., Fukushi Y., Nishimura H. and Mizutani J. (1992), A potent attractant of zoospores of *Aphanomyces cochlioides* isolated from its host, *Spinacea oleracea*. Experientia **48**, 410–414.

Horio T., Yoshida K., Kikuchi H., Kawabata J. and Mizutani J. (1993), A phenolic amide from roots of *Chenopodium album*. Phytochemistry **33**, 807–808.

Islam M. T. and Tahara S. (2001), Survey of physiologically active compounds in traditional medicinal plants regulating the behavior of zoospores of phytopathogenic fungus *Aphanomyces cochlioides*. Proceedings of the International Symposium "Utilization of natural products in developing countries: trends and needs", July 9–14, 2000, University of West Indies, Mona Campus, Kingston (in press).

Jones H. E. H. and Pope G. S. (1960), The action of miroestrol and other estrogens on the reproductive tract of immature female mouse. J. Endocrinol. **20,** 229–235.

King R. J. B. and Manwaring W. I. P. (1974), Steroid Cell Interactions. Butterworths, London.

Krishnan A. V., Stathis P., Permuth S. F., Tokes L. and Feldman D. (1993), Bisphenol A: An estrogenic substances is released from polycarbonate flasks during autoclaving. Endocrinology **132**, 2279–2286.

Leavitt W. W. and Meismer D. M. (1968), Sexual development altered by non-steroidal oestrogens. Nature **218**, 181–182.

Mizutani M., Hashidoko Y. and Tahara S. (1998), Factors responsible for inhibiting the motility of zoospores of the phytopathogenic fungus *Aphanomyces cochlioides* isolated from the non-host plant *Portulaca oleracea*. FEBS Lett. **438**, 236–240.

Morris P. F. and Ward E. W. B. (1992), Chemoattraction of the zoospores of the soybean pathogen, *Phytophthora sojae*, by isoflavones. Physiol. Mol. Plant Pathol. **40**, 17–22.

Rai P. V. and Strobel G. A. (1966), Chemotaxis of zoospores of *Aphanomyces cochlioides* to sugar beet seedlings. Phytopathology **56**, 1365–1369.

Steinmetz R., Mitchner N. A., Grant A., Allen D. L., Bigsby R. M., and Ben J. N. (1998), The xenoestrogen bisphenol A induces growth, differentiation, and c-fos gene expression in the female reproductive tract. Endocrinology **139**, 2741–2747.

Tahara S., Mizutani M., Takayama T. and Ohkawa K. (1999), Plant secondary metabolites regulating behaviour of zoospores of the phytopathogenic fungus *Aphanomyces cochlioides*. Pest. Sci. **55**, 209–211.

Takai Y., Tsutsumi O., Ikezuki Y., Hiroi H., Osuga Y., Momoeda M., Yano T. and Taketani Y. (2000), Estrogen receptor-mediated effects of a xenoestrogen, bisphenol A, on preimplantation mouse embryos. Biochem. Biophys. Res. Commun. **270**, 918–921.

Takahashi H., Sasaki T. and Ito M. (1987), New flavonoids isolated from infected sugar beet roots. Bull. Chem. Soc. Jpn. **60**, 2261–2262.

Takao N., Shimoda C. and Yanagishima N. (1970), Chemical nature of yeast sexual hormones. Develop., Growth Differentiation **12**, 199–205.

Ui T. and Nakamura S. (1963), Sugar-beet black root and its pathogen *Aphanomyces cochlioides*: Pathogenicity and host specificity. Tensai-Kenkyukai-Hokoku **3**, 78–95. (Japanese).

Yokosawa R. and Kuninaga S. (1979), *Aphanomyces raphani* zoospore attractant isolated from cabbage: Indole-3-aldehyde. Ann. Phytopathol. Soc. Jpn. **45**, 339–343.

Yokosawa R., Kuninaga S. and Sekizaki H. (1986), *Aphanomyces euteiches* zoospore attractant isolated from pea root: Prunetin. Ann. Phytopathol. Soc. Jpn. **52**, 809–816.

Yokosawa R., Sekizaki H. and Kuninaga S. (1988), Attractants of *Aphanomyces cochlioides* zoospores contained in sugar beet seedlings. Ann. Phytopathol. Soc. Jpn. **54**, 133–140.

Zentmyer G. A. (1961), Chemotaxis of zoospores for root exudates. Science **133**, 1595–1596.