Factors responsible for inhibiting the motility of zoospores of the phytopathogenic fungus *Aphanomyces cochlioides* isolated from the non-host plant *Portulaca oleracea*

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Abstract In a survey of plant secondary metabolites regulating the behaviour of *Aphanomyces cochlioides* zoospores, we found that root extracts of *Portulaca oleracea* inhibited zoospore motility. Bioassay-directed fractionation of *Portulaca* constituents revealed that the inhibitory activity was dependent on the interaction of two chemically different factors. These were identified as a phenolic compound, *N-trans*-feruloyltyramine, which by itself was active as a zoospore stimulant, and an acidic compound, 1-linoleoyl-2-lysophosphatidic acid monomethyl ester, which had zoospore-repellent activity. When Chromosorb W AW particles coated with a mixture of these pure compounds were bioassayed in Petri dishes, the inhibitory effect on zoospore motility was identical with that caused by root tip or root extracts of *P. oleracea*. Inhibited zoospores rapidly settled to the bottom of the Petri dishes where they initially encysted, and then germinated within 1–2 h. This is the first report of factors which inhibit zoospore motility without killing or bursting the zoospores.

Key words: Zoospore motility; Stimulant; Repellent; Motility inhibiting factor; *Portulaca oleracea*; *Aphanomyces cochlioides*

1. Introduction

Aphanomyces cochlioides is a soil-borne phytopathogenic fungus which is responsible for a root rot disease of spinach (*Spinacia oleracea* L.) and a damping-off disease of sugar beet (*Beta vulgaris* var. *rapa* Dum.). The fungus also infects other species of Chenopodiaceae and Amaranthaceae. The flagellate zoospores of *Aphanomyces* spp. originate from oospores or zoosporangia formed in diseased plant tissues, and swim in the soil water to the roots of uninfected host plants. It is believed that constituents in root exudates attract zoospores to the host plant where they aggregate as a hemispheric mass on the root surface before infection occurs. Compounds such as indole 3-carbaldehyde from cabbage seedlings [1], prunetin from pea seedlings [2], and cochliophilin A (**1**) from the roots of spinach [3] have already been identified as host-specific zoospore attractants of *A. raphani*, *A. euteiches*, and *A. cochlioides*, respectively. In contrast to susceptible plants, it is thought that the roots of non-host plants may exude chemical signals which in some way contribute to resistance. We extracted the aerial parts and roots of 49 wild plants and bioassayed (particle method [3]) these extracts to determine their effect on the motility of zoospores of *A. cochlioides* in water. Out of 98 crude extracts, only the root extract of *Portulaca oleracea* completely inhibited movement of the zoospores, whilst other extracts showed attractant, repellent, stimulant or growth inhibitory activity. Our current research has been focused on the active principles which halt zoospore motility. Fractionation of the *Portulaca* root extracts revealed that the inhibitory activity was dependent on the presence of two chemically different factors. In this paper, we report the isolation and structure elucidation of these two compounds, one of which is an aromatic amide (zoospore stimulant) and the other a lipid (zoospore repellent).

2. Materials and methods

2.1. Preparation of the zoospore suspension

A. cochlioides AC-5 [3] was grown for 7–10 days on corn meal agar (Difco) in a Petri dish (9 cm i.d.) at 20°C. Half of the mycelium-covered agar was then transferred to another Petri dish containing 80 ml of distilled water. To remove nutrients from the agar, the water in the second Petri dish was changed three times (total 240 ml) at intervals of 20 min. The Petri dish containing mycelium and agar, and a final 25 ml of distilled water, was then allowed to stand for 15–24 h at 20°C to promote the release of zoospores. The zoospore concentration was adjusted to ca 1.2×10^5/ml with distilled water immediately before the bioassay was carried out.

2.2. Bioassay

Particles of Chromosorb W AW (80–100 mesh) were used as the carrier of the test compounds. The particles were placed on a watch glass, and onto them was dripped 5 μl of a diethyl ether or ethyl acetate solution of the test compound. Any excess solution on the watch glass was immediately absorbed with a piece of filter paper, and the particles were then air-dried at room temperature. A few of the treated particles were carefully dropped into an aqueous suspension (2.5 ml) of zoospores in a small Petri dish (3 cm i.d.). The behaviour of zoospores around these particles was observed microscopically up to 4 min after addition of the particle(s). Control particles were treated with solvent alone [3,4]. 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 particles treated with any of the active compounds responded in one of the following ways. (i) Attractant activity: relatively large numbers of zoospores aggregated around the particles, moving in a complex zigzag or circular manner, and at increased speed. There was a clear gradient in zoospore density which decreased observably with increasing distance from the particle. (ii) Repellent activity: zoospores would not approach the treated particles which quickly became surrounded by a circular, zoospore-free zone. (iii) Stimulant activity: zoospore movement near the particles increased in speed without any variation in density. (iv) Inhibitory activity: zoospore movement was suddenly halted in an area around the particles. The zoospores finally settled on the bottom of the Petri dish. As the quantity of the

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Abbreviations: EI-MS, electron impact mass spectrometry; EtOAc, ethyl acetate; FD-MS, field desorption mass spectrometry; HR-FAB-MS, high resolution fast atom bombardment mass spectrometry; MeOH, methanol

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various active compounds applied to the particles was increased, these responses became more evident.

2.3. Plant materials

Plant materials used in the present study were collected in and around Hokkaido University. After washing with water, each plant was divided into aerial parts and roots, and these were extracted separately with MeOH. Each MeOH extract was concentrated, and the residue was partitioned between EtOAc and water. The constituents in each fraction were subjected to the particle bioassay using *Aphanomyces* zoospores.

2.4. Methylation of lysophosphatidic acid

1-Oleoyl-2-lysophosphatidic acid sodium salt (12 mg) purchased from Funakoshi Co. Ltd., was dissolved in $CHCl_3$-MeOH, to which was added an excess amount of diazomethane in ether. The resulting mixture was stirred for 30 min at room temperature. When the reaction mixture was chromatographed on silica gel thin-layer plates in $CHCl_3$-MeOH-H_2O 65:25:4, two spots were detected at R_f 0.2 and 0.8. The remaining reaction mixture was concentrated and applied to a silica gel column. Two components were eluted separately from the column using the $CHCl_3$-MeOH-H_2O mixture, and were identified as the corresponding monomethyl (4.0 mg, negative mode FAB-MS, $[M\text{-}H^+]^-$ *m/z* 449) and dimethyl (6.6 mg, FD-MS M^+ *m/z* 464) esters.

2.5. Methanolysis of the repellent from Portulaca oleracea

A small portion of the repellent, ca 0.2 mg, from *P. oleracea* was dissolved in MeOH containing 0.1 N KOH and stirred for 24 h at room temperature. The reaction mixture was acidified (pH < 3.0) with 2 N HCl and developed on silica gel thin-layer plates in hexane-EtOAc 4:1 to give two spots at R_f 0.1 and 0.7, which were respectively confirmed to be unchanged starting material and methyl linoleate by comparison with reference compounds.

3. Results and discussion

3.1. Detection of zoospore inhibitory activity and the agents responsible

As a result of observing the behaviour of *Aphanomyces* zoospores using the particle bioassay method, only EtOAc-soluble material from the root extract of common purslane, *Portulaca oleracea*, clearly inhibited zoospore motility. No inhibitory activity was associated with an extract of the aerial parts of *P. oleracea*, or with extracts of the roots and aerial parts of 48 other plant species. Preliminary extraction and fractionation of the constituents in a few hundred grams of *P. oleracea* roots was next carried out in order to obtain further information on the nature of the inhibitory factor(s). The EtOAc-soluble components from an MeOH extract were suspended in 50% MeOH and washed with hexane (×3). The aqueous MeOH layer was reduced to a low volume in vacuo and extracted successively with ether (×3) and EtOAc (×3). The ether-soluble substances possessing inhibitory activity were partitioned into phenolic+neutral, acidic, and basic fractions in the normal way. The acidic and neutral+phenolic fractions were found to respectively display inhibitory and stimulatory activity, whilst the basic fraction was completely inactive. The acidic fraction was subjected to silica gel TLC in EtOAc-MeOH-H_2O-conc. NH_4OH 60:15:5:1 (2× development), and the plate was then divided into 12 horizontal zones from the origin to the solvent front (13.7 cm). The silica gel in each zone was removed and any compounds were eluted from it with EtOAc. When the eluates were checked for biological activity, none exhibited inhibitory properties. However, eluates from the third zone (4.0–4.9 cm) and the 11th zone (11.3–12.5 cm) showed repellent and stimulant activity, respectively. When these eluates were combined, the zoospore inhibitory activity, characteristic of the crude extract, was restored. Furthermore, the less polar factor from zone 11 was not an acidic substance, but a phenolic component which was probably dispersed into the acidic and neutral+phenolic fractions during ether extraction. The major proportion of this constituent was found in the neutral+phenolic fraction, which showed reasonable zoospore stimulating activity. The characteristic

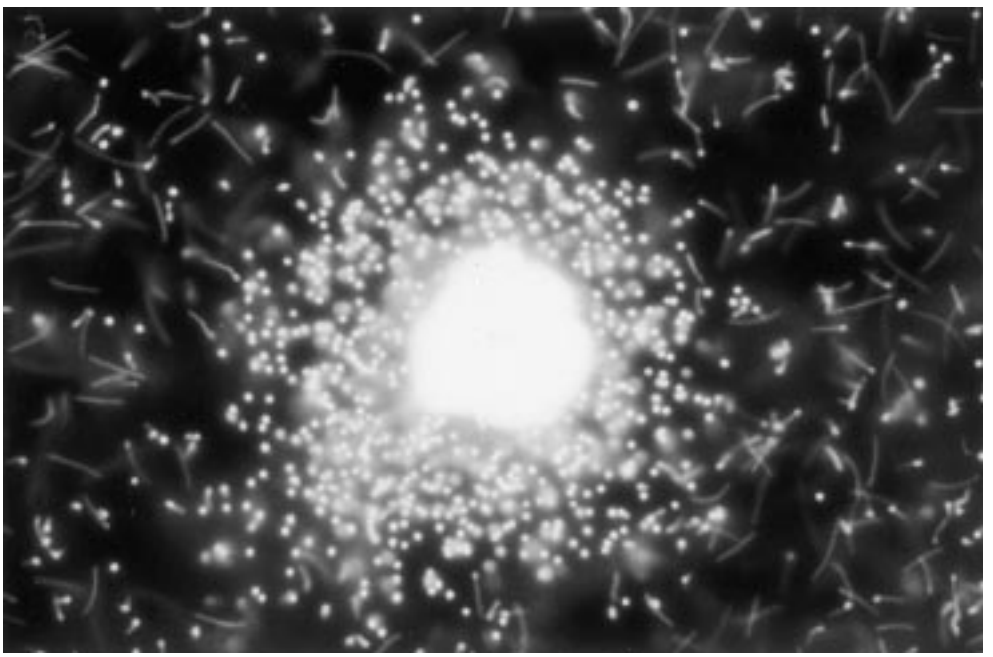
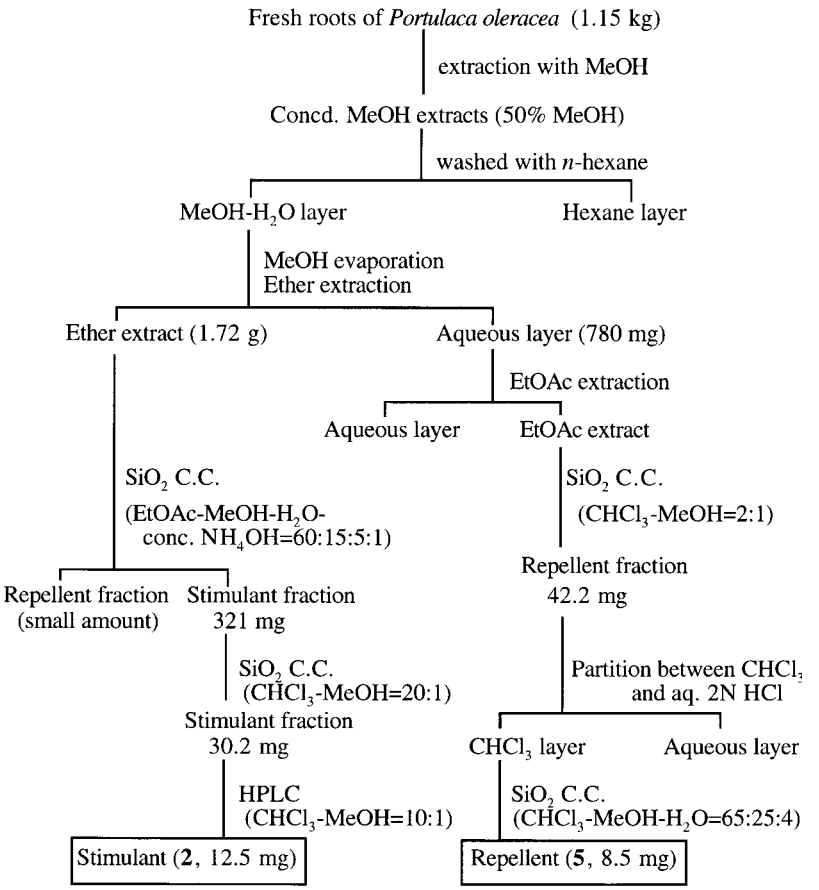


Fig. 1. Photomicrograph of zoospores of *A. cochlioides* after exposure to a mixture of the stimulant, *N-trans*-feruloyltyramine (**2**) and the repellent, 1-linoleoyl-2-lysophosphatidic acid monomethyl ester (**5**) released from a Chromosorb particle treated with a 1000 ppm and a 100 ppm solution of **2** and **5**. Photograph was taken through a microscope with a 90× magnification and an exposure time of 0.5 s. Particle: Chromosorb W AW ca 150–200 μm. Dots close to the particle: inhibited zoospores. Lines in the area remote from the particle: traces of swimming zoospores.



Scheme 1. Isolation procedure for compounds in *P. oleracea* roots exhibiting stimulant and repellent activity on zoospores of *A. cochlioides*. SiO_2 C.C.: silica gel column chromatography.

behaviour of *Aphanomyces* zoospores is shown in Fig. 1, where zoospore movement is inhibited in the area close to a Chromosorb W AW particle treated with a mixture of the stimulant and repellent factors from roots of *P. oleracea*, whilst in areas more remote from the particle, they are still swimming quite actively.

3.2. Isolation of the factors contributing to zoospore inhibition

P. oleracea roots (1.15 kg) were washed, air-dried overnight, and extracted with MeOH. The extract was fractionated as shown in Scheme 1. The ether extract (Scheme 1) was subjected to silica gel (200 g) column chromatography using EtOAc-MeOH-H_2O-conc. NH_4OH 60:15:5:1 to give 12 fractions (100 ml each) in which two active components, a stimulant (fractions 2–4) and a repellent (fraction 6) were detected. Fractions 2–4 were rechromatographed and the stimulant was finally purified by HPLC using an Inertsil column (6.0×250 mm) in $CHCl_3$-MeOH 20:1, flow rate 1 ml/min, to yield 12.5 mg of active compound (t_R ca 16.3 min).

As the amount of repellent in the ether extract was insufficient for further purification, the EtOAc extract (Scheme 1) was used as an alternative source. This extract was initially applied to a silica gel (60 g) column and the repellent was eluted with $CHCl_3$-MeOH 2:1. Fractions 6–8 (40 ml each), which contained the repellent, were combined, the volume reduced to near dryness in vacuo, and the residue redissolved in EtOAc and washed with 2 N HCl. The EtOAc-soluble constituents were further purified by passing through a silica gel Sep-Pak column (3 cc) with $CHCl_3$-MeOH-H_2O 65:25:4 as eluting solvent to give the repellent (8.5 mg) as a spot positive to Dittmer phosphate reagent [5] on silica gel thin-layer plates (R_f 0.2 in the same solvent).

3.3. Characterisation of the zoospore stimulant

HR-EI-MS indicated the empirical formula $C_{18}H_{19}O_4N$, whilst the ^{1}H-NMR spectrum revealed two hydroxyl groups [δ 7.88 (1H, *s*) and δ 8.09 (1H, *s*)], seven aromatic protons [δ 6.7–7.2 (7H)], one methoxy group [δ 3.88 (3H, *s*)], two methylene groups [δ 2.74 (2H, *t*, *J*=7.3 Hz) and δ 3.48 (2H, *q*, *J*=7.3 Hz)], and two olefinic protons [δ 6.47 (1H, *d*, *J*=15.5 Hz) and δ 7.42 (1H, *d*, *J*=15.5 Hz)]. The coupling constant of 15.5 Hz indicated the presence of a *trans*-disubstituted olefinic bond. The detection of protons assignable to a methoxyl, and a hydroxyl group, an olefinic group and a 1,2,4-trisubstituted benzene, as well as an EI-MS fragment at *m/z* 177, strongly indicated the presence of a feruloyl part structure. The remaining four aromatic protons [δ 6.75, *d*, *J*=8.4 Hz and 7.06 (2H, *d*, *J*=8.4 Hz)] and four protons attributable to two methylene groups were immediately assigned to those of tyramine. Most of these signals were very similar to those of *N-trans*-feruloyl-3-*O*-methyldopamine (**4**) and *N-trans*-feruloyl-4-*O*-methyldopamine (**3**) previously isolated from *S. oleracea* [6] and *Chenopodium album* [7]. The stimulant was thus considered to be *N-trans*-feruloyltyramine (**2**).

This identification was confirmed by acylation of commercially available tyramine with ferulic acid in the presence of

N,*N*′-dicyclohexylcarbodiimide to yield *N-trans*-feruloyltyramine (**2**) which possessed physicochemical properties indistinguishable from those of the natural stimulant. The stimulant activity of natural *N-trans*-feruloyltyramine (**2**) towards the zoospores of *A. cochlioides* was also comparable with that observed for the synthetic compound (**2**). The minimum concentration required to cause detectable stimulant activity was ca 1.0×10^{-6} M by the particle method. This activity was 100 times weaker than that of *N-trans*-feruloyl-4-*O*-methyldopamine (**3**) isolated from *C. album* roots [6], which showed both stimulant and attractant activities at a concentration of 1.0×10^{-8} M. *N-trans*-Feruloyltyramine (**2**) has previously been isolated from *Solanum melongena* L. where it appears to be involved in retardation of continuous cropping [8].

3.4. Characterisation of the zoospore repellent

The purified repellent, containing minute amounts of a homologue (or homologues), gave a positive response to the Dittmer test which indicated the presence of a phosphate group in the molecule [5]. In the ^{31}P-NMR spectrum, a phosphorus atom resonated at 4.54 ppm (internal standard: triphenylphosphine). Alkaline methanolysis of the repellent yielded methyl linoleate indistinguishable from an authentic sample. In the ^{1}H-NMR spectrum, signals assignable to two double bonds via one methylene (–CH=CH–CH_2–CH=CH–), and a methyl doublet coupled with ^{31}P via an oxygen atom [δ 3.66 (3H, *d*, *J* = 10.9 Hz) [9] were detected. The molecular formula of the repellent was found to be $C_{22}H_{41}O_7P$ by HR-FAB-MS (negative ion mode: $[M\text{-}H^+]^-$, *m/z* 447). From an analysis of all the available physicochemical data (see Fig. 2), the repellent was considered to have structure **5**.

This structure was confirmed as follows. Commercially available 1-oleoyl-2-lysophosphatidic acid (**6**) was methylated with diazomethane in ether to yield the monomethyl (**7**) and dimethyl (**8**) esters, which were respectively positive and negative in the Dittmer test. Chromatographic and spectroscopic properties of synthesised **7** were in good agreement with those of the natural repellent except for minor features reflecting differences in the fatty acid part of each molecule.

3.5. Biological activity and the possible function of the zoospore inhibiting factors

Compounds **5**–**8** (Fig. 2), in addition to 1,2-dioleoylphosphatidic acid, were tested using the particle bioassay. Commercially available 1-oleoyl-2-lysophosphatidic acid (**6**) possessed repellent activity which was enhanced by monomethylation (**7**). When *Aphanomyces* zoospores were pre-treated with an excess of the natural stimulant *N-trans*-feruloyltyramine (**2**), and then exposed to Chromosorb W AW particles coated with various test compounds, it was found that 1-oleoyl-2-lysophosphatidic acid (**6**, 100 ppm) and its monomethyl ester (**7**, 10 ppm), as well as the natural repellent 1-linoleoyl-2-lysophosphatidic acid monomethyl ester (**5**, 30 ppm), effectively inhibited zoospore motility (Table 1). However, 1-oleoyl-2-lysophosphatidic acid dimethyl ester (**8**) and 1,2-dioleoylphosphatidic acid tested with and without the stimulant showed neither repellent nor inhibitory activity. The bioassay results show that all the compounds possessing repellent activity are monoacylated phosphatidic acid derivatives containing at least one hydroxyl group on the phosphoryl unit.

On the basis of the bioassay results, we conclude that a

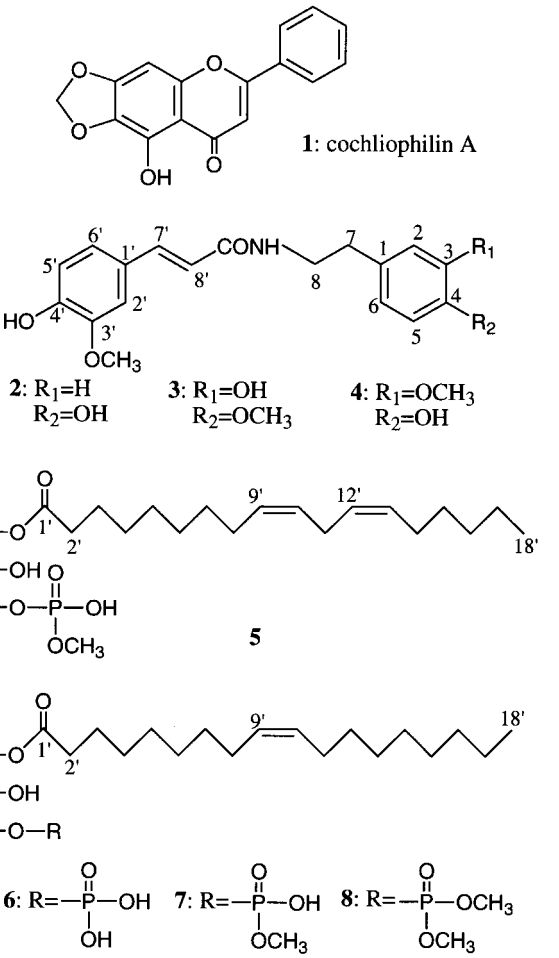


Fig. 2. Structures of compounds mentioned in the text. Physicochemical properties of the natural stimulant *N-trans*-feruloyltyramine (**2**), the natural repellent 1-linoleoyl-2-lysophosphatidic acid monomethyl ester (**5**) and the monomethyl ester of commercially available 1-oleoyl-2-lysophosphatidic acid (**7**) are given below. **2**: Colourless oil. HR-EI-MS: 313.1305 ($[M]^+$, $C_{18}H_{19}O_4N$ requires 313.1315). EI-MS *m/z* (%): 313 ($[M]^+$, 31), 193 (58), 192 (58), 177 (100), 145 (22), 120 (21), 107 (8), 89 (6). UV λmax (MeOH) nm: 222, 292, 319. ^{1}H-NMR δ (acetone-d_6, 270 MHz): 2.74 (2H, *t*, *J* = 7.3 Hz, –CH_2–CH_2–NH–), 3.48 (2H, *q*, *J* = 7.3 Hz, –CH_2–CH_2–NH–), 3.88 (3H, *s*, 3-OCH_3), 6.47 (1H, *d*, *J* = 15.5 Hz, –CH=CH–CO–), 6.75 (2H, *d*-like, *J* = 8.4, H-2 and H-6), 6.82 (1H, *d*, *J* = 8.3 Hz, H-5′), 7.01 (1H, *dd*, *J* = 8.3 and 2.2 Hz, H-6′), 7.06 (2H, *d*-like, *J* = 8.4, H-3 and H-5), 7.14 (1H, *d*, *J* = 2.2 Hz, H-2′), 7.42 (1H, *d*, *J* = 15.5 Hz, –CH=CH–CO–), 7.88 (1H, *s*, 4-OH or 4′-OH), 8.09 (1H, *s*, 4′-OH or 4-OH). The signal for NH was overlapped with other signals at δ 7.0–7.2. **5**: Colourless oil. HR-FAB-MS (negative ion mode, matrix: triethanolamine): 447.2483 ($[M\text{-}H^+]^-$, $C_{22}H_{40}O_7P$ requires 447.2513). FAB-MS *m/z* (%): 447 ($[M\text{-}H^+]^-$, 77), 279 (56), 167 (40), 153 (27), 124 (24), 111 (67), 79 (100), 63 (28). IR νmax (film) cm^{-1}: 3393 (O–H), 2926 (C–H), 1738 (C=O), 1464, 1203, 1051 (P–O–CH_3). ^{1}H-NMR δ ($CDCl_3$, 270 MHz): 0.89 (3H, *t*, *J* = 7.6 Hz, 18′-CH_3), 1.2–1.4 (14H, *m*, –CH_2–), 1.6 (2H, *m*, 3′-CH_2), 2.05 (4H, *m*, 8′ and 14′-CH_2), 2.33 (2H, *t*, *J* = 7.6 Hz, 2′-CH_2), 2.80 (2H, *m*, 11′-CH_2), 3.66 (3H, *d*, *J* = 10.9 Hz, P–O–CH_3), 3.9-4.2 (5H, *m*, glycerol CH_2 and CH protons), 5.36 (4H, *m*, H-9′, 10′, 12′, and 13′). ^{31}P-NMR [$CDCl_3$, 202 MHz, internal standard: $P(Ph)_3$]: 4.54 (br. *s*). **7**: HR-FAB-MS (negative ion mode; matrix, triethanolamine): 449.2668 ($[M\text{-}H^+]^-$, $C_{22}H_{42}O_7P$ requires 449.2670). FAB-MS *m/z* (%): 449 ($[M\text{-}H^+]^-$, 100), 281 (92), 167 (38), 153 (24), 124 (19), 111 (61), 79 (88), 63 (24). ^{1}H-NMR δ ($CDCl_3$, 270 MHz): 0.88 (3H, *t*, *J* = 7.6 Hz, 18′-CH_3), 1.2–1.4 (20H, *m*, –CH_2–), 1.61 (2H, *m*, 3′-CH_2), 2.01 (4H, *m*, –CH_2–CH=), 2.34 (2H, *t*, *J* = 7.6 Hz, 2′-CH_2), 3.76 (3H, *d*, *J* = 10.9 Hz, P–O–CH_3), 3.9–4.2 (5H, *m*, glycerol CH_2 and CH protons), 5.34 (2H, *m*, –CH=).

Table 1
Zoospore inhibitory activity of lysophosphatidic acids and related compounds when mixed with the natural zoospore stimulant *N-trans*-feruloyltyramine (**2**)

Test compound	Zoospore inhibitory activity (ppm)						
	1000	300	100	30	10	3	1
Portulaca repellent (**5**)	NT	+	+	+	−	−	NT
1-Oleoyl-2-lysophosphatidic acid (**6**)	+	+	+	−	−	NT	NT
1-Oleoyl-2-lysophosphatidic acid monomethyl ester (**7**)	NT	NT	+	+	+	−	−
1-Oleoyl-2-lysophosphatidic acid dimethyl ester (**8**)	−	NT	NT	NT	NT	NT	NT
1,2-Dioleoylphosphatidic acid	−	NT	NT	NT	NT	NT	NT

+: inhibited; −: no response; NT: not tested.
Chromosorb W AW particles coated with each test solution were added to a suspension of zoospores which had first been stimulated by exposure to particles treated with 1000 ppm of stimulant **2**.

mixture of the stimulant, *N-trans*-feruloyltyramine (**2**), and the repellent, 1-linoleoyl-2-lysophosphatidic acid monomethyl ester (**5**), in *Portulaca* root is responsible for inhibiting the motility of zoospores of *A. cochlioides*. The behaviour of zoospores treated with a mixture of these pure compounds was very similar to the effect observed when zoospores were exposed to segments of fresh roots of *P. oleracea*. This result suggests that both these active compounds, and especially 1-linoleoyl-2-lysophosphatidic acid monomethyl ester **5**, are not artefacts, but natural compounds possessing important in vivo functions which remain to be fully elucidated. In an earlier study [3], the flavone, cochliophilin A (**1**), was isolated as a potent zoospore attractant of *A. cochlioides* from the roots of the host plant spinach (*S. oleracea*). When bioassayed using the particle method, it was found that cochliophilin A (**1**), like the stimulant *N-trans*-feruloyltyramine (**2**), could act together with the natural repellent 1-linoleoyl-2-lysophosphatidic acid monomethyl ester (**5**) to completely inhibit zoospore motility. Under the microscope, the treated zoospores were first seen to become stationary, and then settle at the bottom of the Petri dish where they encysted to give cystospores. These cystospores germinated within 1–2 h, although germination would not normally be expected in the absence of a host plant. On the roots of spinach, for example, the attracted zoospores assembled to give a mass of cystospores prior to germination and penetration of the host tissues.

This is the first report on the inhibition of zoospore motility as a result of the interaction of a zoospore stimulant (*N-trans*-feruloyltyramine, **2**) and a repellent (1-linoleoyl-2-lysophosphatidic acid monomethyl ester, **5**). The data also indicate a new biological action for lysophosphatidic acid, derivatives of which are already known to exhibit chemoattractant effects on the amoeba, *Dictyostelium discoideum* [10]. The biochemical properties of lysophosphatidic acids are described in detail in the latest review [11]. Hydroxylated *N*-cinnamoyl-β-phenylethylamine derivatives including *N-trans*-feruloyltyramine (**2**) are relatively widespread in higher plants [12]. Their physiological functions are of general interest because their biosynthesis from the corresponding acyl-CoA and amine derivatives, under the influence of enzymes such as tyramine feruloyl transferase, is stimulated in response to pathological infection [13,14].

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