Monogonanta exhibit cyclical parthenogenesis where asexual reproduction predominates but sexual reproduction can occur[11]. In parthenogenetic reproduction, the reliability of producing new generations takes precedence over obtaining genetic variation inherent in sexual reproduction. Parthenogenesis occurs in certain members of the closely related Phylum Nematoda and exists mainly in individuals living in relatively stable environments and therefore not requiring high degrees of genetic variability within populations[12]. Bdelloids do not fit that category, however, because the terrestrial forms are in a constantly changing environment. Maynard-Smith stated that 'a group which does wholly abandon sexual reproduction has a limited evolutionary future'[13]. Although he admitted that bdelloid rotifers represented an anomaly he concluded that, despite the bdelloids, parthenogens are doomed to an early extinction because of lack of evolutionary potential. Other authors have stated that the entire loss of meiotic sexuality has not been tolerated[14].

Up until now, it appeared that these statements might well have been correct even for the bdelloids since with a fossil record of less than 8000 years[1], the bdelloids might be considered a relatively recent group with limited potential for survival. We now know, however, that bdelloids have existed for a considerably longer time than previously thought and that their parthenogenetic mode of development has, at least in some lines, been successful. After extensively reviewing the fossil record for evidence of behavior and coevolution, Boucot[15] concluded that the data show that, on the basis of functional morphology, the behavior of fossil animals, including the mode of fertilization and sexual development, remains constant. He reported that it is reasonable to assume that the behavior of fossil species will be the same as extant forms, almost always down to the taxonomic level of family and certainly at the higher categories. Therefore we conclude that the fossil bdelloids reported here reproduced parthenogenetically. Of course the possibility that certain lineages of fossil bdelloid rotifers possessed an amphimictic or cyclic type of reproduction cannot completely be ruled out. However, the large body of data attesting to the behavioral fixity of fossil organisms does not support this contention. Success of the parthenogenetic bdelloids could possibly be explained by possession of a very plastic genotype, as suggested by Lynch[16] or by still unknown means of exchanging genes as speculated by Maynard-Smith[13].

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A potent attractant of zoospores of *Aphanomyces cochlioides* isolated from its host, *Spinacia oleracea*

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Abstract. A highly potent attractant of zoospores of *Aphanomyces cochlioides*, a causal fungus of the root rot disease of spinach (*Spinacia oleracea*), was isolated from spinach roots, and its structure was determined by spectroscopic evidence and chemical synthesis as cochliophilin A (5-hydroxy-6,7-methylenedioxyflavone, **1**). A chromosorb particle prepared by soaking in solution of **1** showed a potent attracting activity toward the zoospores using concentrations of **1** above 10^{-9} or 10^{-10} M.
Key words. Aphanomyces cochlioides; *Spinacia oleracea*; zoospore; attractant; cochliophilin A.

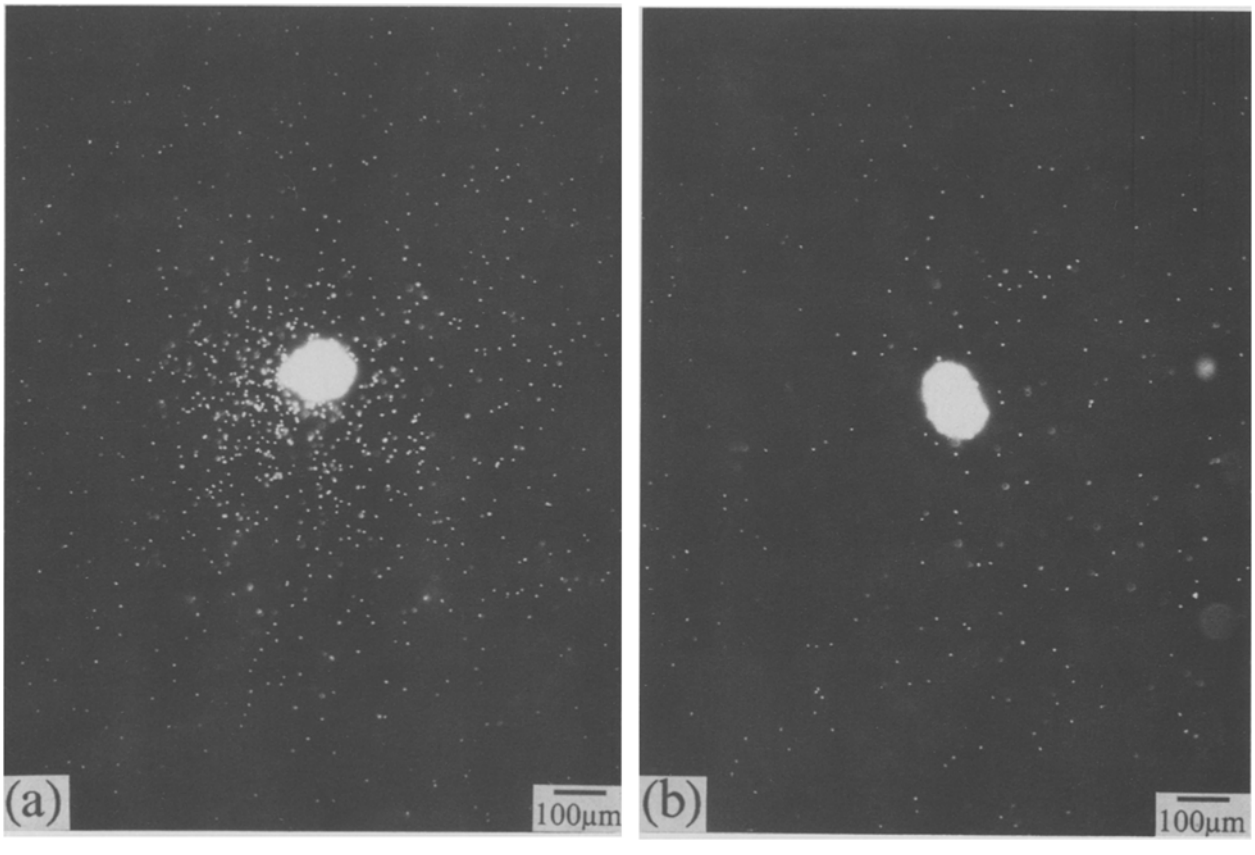
Aphanomyces cochlioides, one of the plant pathogenic fungi, is a causal agent of the root rot disease of spinach (*Spinacia oleracea* L.) and the damping-off disease of sugar beet (*Beta vulgaris* L.), and infects some other species of Chenopodiaceae. Flagellate zoospores of *Aphanomyces* spp. originate from oospores and from zoosporangia formed asexually in diseased tissue, and swim in the soil water. It is considered that when zoospores infect the host plant roots they are initially attracted to exudates from these roots [1–3]. Zoospores of *A. raphani* Kend.[1], which infect Cruciferae, are attracted to the hypocotyls of cabbage seedlings, and zoospores of *A. euteiches* Drechs.[2], which infect Fabaceae, are attracted to the root caps of pea. As attractants of *A. raphani* and *A. euteiches* zoospores, indole-3-aldehyde[4] (**2**) has been identified from cabbage seedlings and prunetin[2] (**3**) from pea seedlings. Rai and Strobel[3] showed that zoospores of *A. cochlioides* were attracted to an organic acid fraction and a neutral fraction (i.e., gluconic acid, fructose and glucose), which were exuded from the roots of sugar beet seedlings. Yokosawa et al.[5] showed that NaCl, KNO_3 and $NaNO_3$ from sugar beet seedlings attracted zoospores of *A. cochlioides* at concentrations between 10^{-2} and 10^{-3} M. Furthermore, *N*-[2(4-hydroxy-3-methoxyphenyl)ethyl]ferulamide[6] (**4**) was also isolated as an active compound. Recently we have found that the roots of adult spinach contain a substance which shows a potent attracting activity for the zoospores of *A. cochlioides*. This report describes the isolation and identification of the attractant from spinach roots, and the evaluation of the attracting activity.

Materials and methods

Fungi: A. cochlioides AK-1 and AC-5 were isolated from the soil of spinach and beet fields, respectively.

Preparation of zoospore suspension: A. cochlioides was grown for 3–4 days on a corn meal agar (Difco) plate at 20 °C. Half of the agar plate, covered with mycelia, was transferred to a petri dish containing 40 ml of distilled water. To remove nutrients from the agar plate, the water in the petri dish was exchanged three times for 40 ml of distilled water at intervals of 30 min. Finally, the petri dish containing 25 ml of distilled water was allowed to stand for 15–24 h at 20 °C to promote the releasing of the zoospores. Zoospore concentration was adjusted to about 10^4/ml with distilled water just before the bioassay.

Bioassay: A few particles of chromosorb WAW (80–100 mesh) as a carrier of test compound were set on a watch glass. 5 µl of a solution of the test compound,



Evaluation of attracting acitivity for *Aphanomyces cochlioides* zoospores. Chromosorb particles are placed in a suspension of the zoospores.

a A particle prepared by soaking in 1.0×10^{-8} M cochliophilin A. *b* A control particle treated with pure solvent. Dark field.

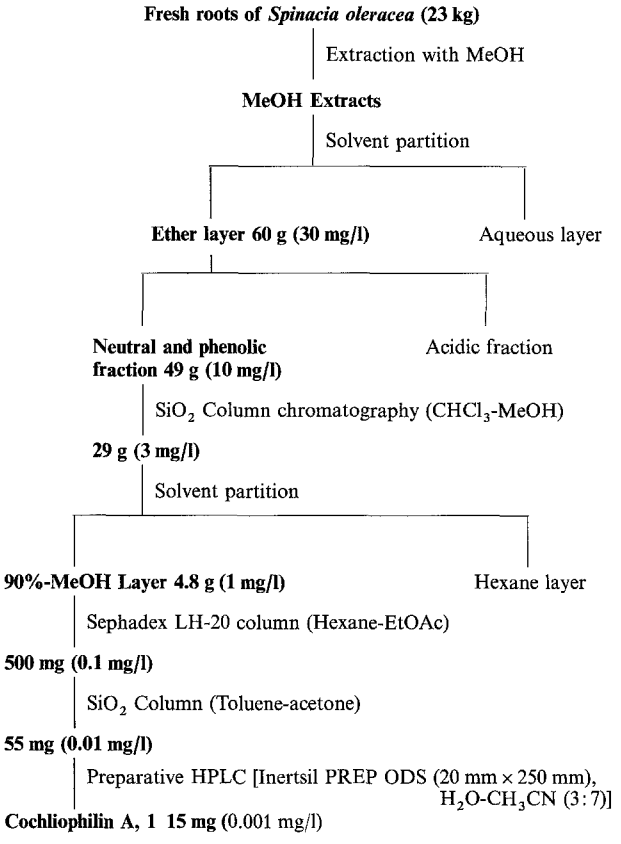
adjusted to an appropriate concentration, was dripped on to the particles. The excess solution on the glass was immediately absorbed with a piece of filter paper, then the particles were air-dried at room temperature. A few of these particles were dropped into the zoospore suspension in a small petri dish on a microscope stage, and the behavior of zoospores around the particles was observed after 1 min. As a control, particles treated only with solvent were used.

With particles treated with a non-active substance, the movement of zoospores was monotonous and straightforward and the speed was not changed. On the other hand, with active substances the movement was in a zigzag or circle; it was complex, and increased in speed. As the quantity of active component applied to the particle was increased, more zoospores collected around the particle. The zoospore density shown in the figure (a) was regarded as a standard for defining the activity, and the minimum concentration showing this density was determined by consecutive dilution of the sample solution. The relative activity of the sample was given by this minimum concentration.

Results

Isolation of attractant: The fractionation procedure used, guided by the bioassay with *A. cochlioides* AK-1, is illustrated in scheme 1. Fresh roots of spinach (*Spinacia oleracea* L. cultivar Solomon) were collected in Sapporo at harvest-time (in November, 1989). The air-dried roots (23 kg) were extracted with methanol. The concentrated extract was partitioned between water (adjusted at pH 3 with 2N HCl) and ether, and then washed with 5%-$NaHCO_3$. The concentrated ether layer yielded 49 g of neutral-phenolic constituents with a relative activity of 10 mg/l. This fraction was chromatographed on a silica gel column and eluted with a chloroform-methanol gradient. Active fractions, eluted with 0–1% methanol in chloroform, were partitioned between 90% aqueous methanol and *n*-hexane. The active aqueous methanol layer was concentrated, and the residue was applied to Sephadex LH-20 column chromatography using a hexane-ethyl acetate gradient as an eluent. The active fraction, eluted with 25–100% ethyl acetate in hexane, was efficiently rechromatographed on a silica gel column by eluting with a toluene-acetone gradient. The active principle was eluted with 1–2% acetone in toluene. Final purification was achieved by HPLC on a C_{18} column (Inertsil PREP ODS, 20 mm i.d. × 250 mm) using acetonitrile-water (7:3) as a mobile phase to yield 15 mg of the active compound **1** (6.5×10^{-5}% total yield). This compound was named cochliophilin A.

Structural determination of cochliophilin A[7]: The UV λ_{max} at 276 and 317 nm suggested that **1** had a flavone structure. In the ^{1}H-NMR spectrum, the presence of a singlet signal at δ 6.68 attributable to the flavone H-3 also supported its structure. Signals of the aromatic protons at δ 7.87 (2H, m) and δ 7.54 (3H, m) indicated that

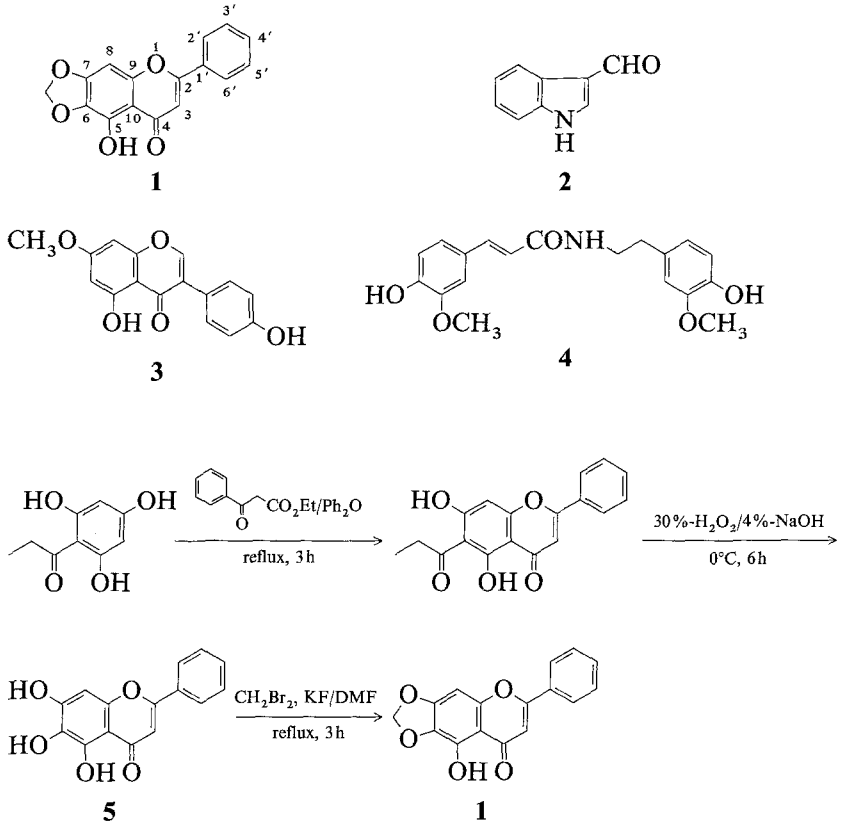


Scheme 1. Isolation procedure for attractant of *Aphanomyces cochlioides* zoospores from roots of *Spinacia oleracea*. The concentration in parentheses gives the relative activity.

the ring B was unsubstituted. One proton signal at δ 12.10 was assignable to a chelated hydroxyl group at C-5. The singlet at δ 6.10 (2H) is characteristic of a methylenedioxy group. Compound **1** showed a positive response to the Gibbs test, which indicated that 8-position was unsubstituted[8]. Therefore, the methylenedioxy group was allocated to C-6 and C-7. Thus, cochliophilin A is identified as 5-hydroxy-6,7-methylenedioxyflavone (**1**).

Synthesis of cochliophilin A: Cochliophilin A was synthesized as shown in scheme 2. 5,6,7-Trihydroxyflavone (**5**), prepared by the method of Agasimundin et al.[9], was transformed into the corresponding 6,7-methylenedioxy derivative according to Iinuma et al.[10]. The physicochemical properties of synthesized cochliophilin A were in good agreement with those of natural **1**.

Attracting activity of 1: The activity of **1** toward the zoospores of *A. cochlioides* (AC-5 and AK-1) was tested in the range of 10^{-7}–10^{-11} M (table). Zoospores of AC-5 were more sensitive than those of AK-1 at any concentration. Zoospores of AK-1 and AC-5 aggregated within a few seconds after chromosorb particles were dropped into the suspension, when the particles had been treated with **1** at the concentration of 10^{-7}–10^{-8} M (for AK-1) and 10^{-7}–10^{-9} M (for AC-5). At the concentration of 10^{-7} M, the zoospore aggregation lasted for over



Scheme 2. Synthesis of cochliophilin A (**1**)

60 min. Even with concentrations of 1.0×10^{-9} M and 1.0×10^{-10} M, the AK-1 and AC-5 zoospores, respectively, showed the typical aggregation, but the density was lower than that shown in the figure (a).

Discussion

For zoosporic plant pathogens, attraction has an important role in increasing the possibility for the pathogen to encounter the host plant. Chemotaxis is known to play a part in the relationship between *A. cochlioides* and its host. More knowledge about the nature of the attractant will increase our understanding of the infection mechanism.

Attracting activity of cochliophilin A (**1**) to zoospores of *Aphanomyces cochlioides*[a]

Concentration (M)	Strains of *A. cochlioides* AK-1	AC-5
1.0×10^{-7}	++	++
3.0×10^{-8}	++	++
1.0×10^{-8}	++	++
3.0×10^{-9}	++	++
1.0×10^{-9}	+	++
3.0×10^{-10}	+	++
1.0×10^{-10}	–	+
3.0×10^{-11}	–	–

[a] Attractive activity at 1 min after dropping the particles into the zoospore suspension. ++ shows activity which is equal to or stronger than that shown in the figure; + shows activity which is weaker than that shown in the figure; – shows no activity.

In the present study, cochliophilin A (**1**) was isolated and found to be a powerful attractant. When bioassays were performed during the isolation procedure the activity was almost proportional to the weight of the sample tested, therefore it is clear that the activity of the crude ether extract was due to compound **1** (scheme 1). The behavior of the zoospores toward cochliophilin A is similar to their response to the surface of spinach roots. Therefore, it may be concluded that cochliophilin A plays an essential role in the attracting phenomenon.

Cochliophilin A has previously been isolated only from sugar beet roots infected with *Rhizoctonia solani*[11], and it showed no growth inhibition against *A. euteiches* or *R. solani*. Flavones containing the 6,7-methylenedioxy group have been found so far only in the taxonomic groups, such as Chenopodiaceae[12,13], Polygonaceae[14], Solanaceae[15], Rutaceae[16] and Amaranthaceae[17]. Although the role of compound **1** in plants is as yet unknown, it is interesting that a substance contained in the host plant should be used by the pathogen as a kairomone. It is possible that this compound also plays a role in the host specificity of *A. cochlioides*.

A solution of **1** shows activity at the low level of 10^{-9}–10^{-10} M. This value is the concentration of the sample solution used to prepare the chromosorb particles in our bioassay, so the actual quantity in the solution around the particle during the bioassay must be far smaller. Fur-

ther investigations on the quantity of cochliophilin A exuded from spinach roots, and the development of a quantitative bioassay, are under way.

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7 Cochliophilin A (5-hydroxy-6,7-methylenedioxyflavone, **1**). Pale yellow needles. HR-EI-MS: 282.0524 ($C_{16}H_{10}O_5$ calcd. 282.0528). EI-MS *m/z* (%): 282 (M^+, 100), 253 (5.4), 224 (16.7), 180 (12.6), 102 (6.4). UV λ_{max}(MeOH) nm: 276, 317. ^{1}H-NMR δ ($CDCl_3$, 500 MHz): 6.10 (2H, s, O-CH_2-O), 6.60 (1H, s, H-8), 6.68 (1H, s, H-3), 7.54 (3H, m, H-3′,4′ and 5′), 7.87 (2H, m, H-2′ and 6′), 12.70 (1H, s, 5-OH). ^{13}C-NMR δ ($CDCl_3$, 125 MHz): 89.5 (C-8), 102.7 (O-CH_2-O), 105.5 (C-3), 107.8 (C-10), 126.2 (C-2′ and 6′), 129.1 (C-3′ and 5′), 130.1 (C-6), 131.2 (C-1′), 131.9 (C-4′), 142.2 (C-5), 153.3 (C-9), 154.1 (C-7), 164.0 (C-2), 183.0 (C-4).
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Nest cell lining of the solitary bee *Hylaeus bisinuatus* (Hymenoptera: Colletidae)

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Abstract. The nest cell lining of *Hylaeus bisinuatus* (Hymenoptera: Colletidae) was shown by high-resolution solid-state [^{13}C]NMR to be composed of lipid polymer and protein. The lipid polymer was shown by reduction and subsequent GC/MS analysis to be comprised of ω-hydroxy fatty acids (C_{20}, C_{22}, C_{24} and C_{26}) and fatty alcohols (C_{16} to C_{30}). The protein portion of the lining had a silk-like amino acid composition.
Key words. Solitary bees; lipid polymer; silk; CP/MAS ^{13}C NMR; Hymenoptera; Colletidae; *Hylaeus bisinuatus.*

The earthen walls of nest cells of most species of ground-nesting bees are endowed with a thin, hydrophobic lining or membrane[1–3]. Exceptions include members of some genera of the andrenid subfamily Panurginae that apparently lack nest cell linings[4] and ground-nesting leaf-cutter bees (Megachilidae) that line their nest cells with cut leaf fragments like their stem-nesting relatives[5]. Lining membranes are produced by adult nesting females prior to provisioning the cell with pollen and nectar. Linings have been variously described as waxy, silken, varnish-like or cellophane-like[1,2,6]. A few taxa, such as *Macropis*[7] and *Centris*[8], collect floral oils or plant resins, respectively, which they utilize in the construction of their cell linings. Chromatographic and spectroscopic analyses of the nest cell linings of most other representative species of ground-nesting bees have positively implicated the lipoidal secretion of the hypertrophied abdominal Dufour's gland as the glandular source of the nest cell lining[9–15]. As no species of the related sphecid wasps have been reported to construct a nest cell lining, secreted nest cell linings of bees may prove to be a critical, derived taxonomic character that unifies the bees (Apoidea) as a single evolutionary lineage.

The cellophane-like nest cell linings of species of the Colletidae, long considered the most primitive family of bees, were until recently thought to be comprised of secretions from thoracic salivary glands or mandibular glands. Microscopic examination of cell linings of the colletid genera *Hylaeus*[16], *Colletes*[11,12], and *Ptiloglossa*[17] has revealed the variable presence of fiber-like