Original Article

Effects of different classes of attractants, cochliophilin A and *N*-(*E*)-feruloyl-4-*O*-methyldopamine, on the response of *Aphanomyces cochlioides* zoospores in their chemoattraction and activation of motility linked with intracellular cAMP

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Cochliophilin A (5-hydroxy-6,7-methylenedioxyflavone, **1**) and *N*-(*E*)-feruloyl-4-*O*-methyldopamine (**2**) are naturally occurring host-specific chemoattractants for *Aphanomyces cochlioides* zoospores. In a cross-competition assay, compound **1** (4 fmol) applied to diatomite particles clearly attracted *A. cochlioides* zoospores in an aqueous solution of excessive compound **2** (1×10^{-6} M) that could mask a concentration gradient of **1** dispersed from the particles. Similarly, **2** (40 fmol) on particles also attracted *A. cochlioides* zoospores in an aqueous solution of **1** (1×10^{-6} M). In addition, compound **2** on the particles did not attract the zoospores in an aqueous solution containing 1×10^{-6} M NADP⁺, while **1** clearly attracted zoospores in the same solution. These results allowed us to speculate that compounds **1** and **2** do not share receptors. The chemosensory system for **1** is probably for host recognition of *A. cochlioides* zoospores, while the system for **2** is linked to cell differentiation. © Pesticide Science Society of Japan

Keywords: *Aphanomyces cochlioides* zoospore chemoattractant 5-hydroxy-6,7-methylenedioxyflavone *N*-(*E*)-feruloyl-4-*O*-methyldopamine cross-competition assay host-recognition system.

Introduction

Aphanomyces cochlioides is a Peronosporomycetes (Oomycota) phytopathogen specifically infecting beet (*Beta vulgaris*),¹⁾ spinach (*Spinacia oleracea*),²⁾ and goosefoot (*Chenopodium album*)³⁾ of the family Amaranthaceae (Chenopodiaceae in the Engler system). In the infection event, the important process of host recognition by *A. cochlioides* secondary zoospores and those of other Peronosporomycetes is mediated by specific chemoattractants exuded from the roots of the host plants.⁴⁾ Due to the establishment of a particle method by Horio *et al.*⁵⁾ for bioassay of *A. cochlioides* zoospore chemotaxis, cochliophilin A (5-hydroxy-6,7-methylenedioxyflavone, **1**)^{5–7)} and *N*-(*E*)-feruloyl-4-*O*-methyldopamine (**2**)^{3,5)} have been isolated and characterized as the host-specific chemoattractants of *A. cochlioides* zoospores. As source plants of compound **1**, sugar beet (*B.*

vulgaris),⁶⁾ spinach (*S. oleracea*),⁵⁾ goosefoot (*C. album*)³⁾ and cockscomb (*Celosia cristata*)⁷⁾ have been reported, and all the **1**-containing plants are susceptible hosts of *A. cochlioides*. In contrast, compound **2** and its analogs, such as *N*-feruloyl-4-*O*-methyltyramine, have been isolated from the roots of hosts *B. vulgaris*⁸⁾ and *C. album*,³⁾ while many other non-host Amaranthaceae plants, including *Amaranthus gangeticus*,⁹⁾ *Portulaca oleracea*,¹⁰⁾ and *Achyranthes ferruginea*,¹¹⁾ also contained the amide-type chemoattractants. Moreover, *Tinospora tuberculata* (Menispermaceae),¹²⁾ *Actinodaphne longifolia* (Lauraceae),¹³⁾ and *Aristolochia gehrtii* (Aristolochiales)¹⁴⁾ have been reported to contain **2** and related compounds.

The naturally occurring chemoattractants **1** and **2** isolated from the host plants are different classes of chemicals, and the reason for these two types of attractants in the host plants remains a mystery. Using the particle method, diatomite carrier particles of Chromosorb® A/WA (60–80 mesh, Shinwa Chemical Industries Co., Kyoto, Japan) that were soaked in an EtOAc solution of an active compound and immediately air-dried were floated on a zoospore suspension.^{4,5)} An effective amount of the active substance applied to the particles as an EtOAc solution at-

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Fig. 1. Chemical structures of naturally occurring chemoattractants and agonistic compounds used in this study. Compounds **1** and **2** are naturally occurring chemoattractants associated with host recognition of *Aphanomyces cochlioides* zoospores. In the particle method using a 1×10^{-6} M solution of a test compound dissolved in EtOAc, only tyrphostin B56 (**6**) showed a ++ attractant activity toward *A. cochlioides* zoospores among several tyrphostin derivatives commercially available. Also, (*E*)-methyl ferulate (**7**) showed a ++ attractant activity in the same bioassay system, in contrast to inactive (*E*)-ferulic acid with the free carboxyl group.

tracts the swimming zoospores, and cell aggregate forms around the particles. In this bioassay method, the flavone-type chemoattractant **1** showed hypersensitive zoospore-attracting activity in the range of 1×10^{-7} to 10^{-9} M applied to the carrier particles. On the other hand, the feruloyl amide-type attractant **2** was less active than **1** and showed clear attraction in the range of 1×10^{-5} to 10^{-7} M solution in EtOAc.⁵ The non-substitution of B-ring and 5-hydroxylation at A-ring are known as partial structures necessary for the attracting activity. Therefore, many analogous compounds of **1**, including 5-hydroxy-6,7-dimethoxyflavone (**3**), 5,7-dihydroxyflavone (chrysin, **4**), and 5,6,7-trihydroxyflavone (**5**), showed agonistic activity toward *A. cochlioides* zoospores.¹⁵ Conversely, a structural variation of compound **2**, which is widely distributed throughout several plant families other than the Amaranthaceae, suggests a relatively narrow attracting activity toward zoospores.

Applying $^3\text{H}_2\text{O}$ to diatomite particles, Takayama *et al.* measured the average volume applicable to these porous particles (approximately $100 \mu\text{m}$ diameter), and *ca.* 4 nL was calculated to be the void volume per particle.¹⁶ In addition, they measured the amounts of lipophilic substances applied to diatomite particles that were released into the aqueous hemisphere around the particles using ^3H -labeled ferulic acid and benzaldehyde.¹⁶ Due to agonistic or antagonistic activities of flavonoids toward purine-type signal messengers including cyclic AMP (cAMP), we also examined the effects of purine derivatives in aqueous solutions have on the behavior of zoospores in the cross-competition assay. In this paper, we describe the results of our bioassays on flavone-type **1** and feruloyl amide-type **2** and fur-

ther discuss their distinguishable roles in the infection process of *A. cochlioides*.

Materials and Methods

1. Chemical compounds

Chemical compounds (**1**, **2**, 5-hydroxy-6,7-dimethoxyflavone **3**, 5,7-dihydroxyflavone (chrysin, **4**), and 5,6,7-trihydroxyflavone **5**) were commercially available (Wako Pure Chemical Industries, Ltd., Osaka, Japan) or synthesized as described previously.^{3,5,15} A tyrosine kinase inhibitor, tyrphostin B56 (**6**)¹⁷ and methyl ferulate (**7**) were purchased from Funakoshi (Tokyo, Japan) and TCI (Tokyo, Japan), respectively (Fig. 1). Purine derivatives tested were adenine (**8**, purchased from Kanto Chemical Co., Tokyo, Japan), guanine (**9**, a product of MP Biomedicals, LLC, Santa Ana, CA), and NADP⁺ (**10**, purchased from Oriental Yeast Co. Ltd., Tokyo, Japan). Both forskolin (**11**), a diterpene-type adenylyl cyclase activator,¹⁸ and 9-(2-tetrahydrofuryl)-adenine (SQ22536, **12**), a purine-type adenylyl cyclase inhibitor,¹⁹ were from Sigma-Aldrich (St. Louis, MO, USA). The chemical structures of all the compounds used in this study are shown in Fig. 2.

2. Preparation of zoospore suspension in aqueous solutions containing test compounds

For preparation of *A. cochlioides* zoospores, half of a cornmeal agar plate (9 cm i.d. Petri dish) was inoculated with *A. cochlioides* mycelia, cultured for 5–10 days, and then soaked in 80–90 mL reverse osmosis (RO) water for 20 min. The water was then discarded by decantation. This washing process was re-

Fig. 2. Chemical compounds used in this study as purine derivatives and adenylate cyclase activator and inhibitor.

peated three times, at which point 20 mL fresh water was added. The dish was kept at 20°C for 20 hr to induce zoospore production.⁵⁾ A given chemical compound, initially dissolved in DMSO or acetone as a 1×10^{-3} M solution, was diluted 1,000-fold with 100 mL RO water to use in the final aqueous solution for zoospore production.

For preparation of zoospore suspensions containing a test compound, *A. cochlioides* mycelia on cornmeal agar plates were washed only twice with RO water, then washed with 40 mL of an aqueous solution containing the test compound at 1×10^{-6} M. Another 40 mL portion of the same solution was then added to the mycelia for the final aqueous solution, allowing emergence of the zoospores from zoosporangia in the mycelial colony. In the 1×10^{-6} M aqueous solutions of the test compounds, including **1**, **2**, and purine derivatives, significant numbers of swimming zoospores were observed under a light microscope (Olympus IX70, Olympus Co., Tokyo, Japan).

3. Bioassay for cross-competition between flavone-type and feruloyl amide-type active compounds

For competition assays, the particle method previously described was modified as follows: porous diatomite particles of Chromosorb® W/AW soaked in 1×10^{-5} to 10^{-8} M solutions of **1** or **2** and immediately air-dried were floated on the zoospore suspension, which was nearly saturated with the test compounds. Responses of the *A. cochlioides* zoospores around the particles were then observed under a light microscope (100 to 200 $\times$).^{5,10)} When the particles carrying **1** or **2** attracted *A. cochlioides* zoospores comparably to the control (RO water only), it was regarded as highly positive (++). In particles highly positive for cell aggregation (++), encysted zoospores formed large, thick cell aggregates around the particle within 1 hr, which was the same response as in the control. When zoospores were obviously attracted to the particle but at lower population numbers

Fig. 3. Responses of *Aphanomyces cochlioides* zoospores to chemoattractant-applied diatomite particles in the particle method. Attractant activity and cell aggregation of *A. cochlioides* zoospores in the particle method were defined in these photographs: ++ (highly active), + (active) and - (inactive) for attraction and aggregation of *A. cochlioides* zoospores. Attraction was generally evaluated within 10 min, while aggregation of the zoospore around the particle was observed after 1 hr.

than (++), it was defined as positive (+). Cell aggregates judged as positive (+) formed thin or irregularly shaped plaques. No or only ambiguous attraction without any cell aggregation was classified as inactive (-) (Fig. 3).

Two types of natural chemoattractants (**1** and **2**) and their agonist-like compounds (flavone types (**3**, **4**, and **5**) and feruloyl types (**6** and **7**)) were combined in the bioassay system. Swimming zoospores were produced for competition assays in aqueous solutions nearly saturated (1×10^{-6} M) with the naturally occurring attractant **1** or **2** or agonist-like compound **3**, **4**, **5**, **6**, or **7**. In the bioassay system, responses of *A. cochlioides* zoospores

in an aqueous solution containing 1×10^{-6} M of a test compound to a particle carrying **1** and **2** were observed in a short period (10 min) and a long period (1 hr or more). Adenine (**8**), guanine (**9**), and NADP⁺ (**10**) at 1×10^{-6} M were also used as solutes in aqueous solutions. Compound **10** was selected because nicotinamide contained as a moiety of **10** affected cell differentiation of *A. cochlioides* zoospores.²⁰⁾ The third washing of *A. cochlioides* mycelia followed by zoospore production was done using these solutions. The swimming zoospores in each solution were tested for chemotactic activity toward compounds **1** and **2** on the particles. After 1 hr of incubation, formation of cell aggregates was also observed.

4. Effect of an adenylate cyclase activator and inhibitor on the response and behavior of *A. cochlioides* zoospores

Aqueous solutions of the adenylate cyclase activator forskolin (**11**) and the adenylate cyclase inhibitor SQ 22536 (**12**) were prepared at a final concentration of 100 μ M.^{21,22)} Aliquots of 50 mM **11** (200 μ L) and 100 mM **12** (100 μ L), both dissolved in DMSO, were each diluted with 100 mL Milli-Q water and then used for the third washing of *A. cochlioides* mycelia followed by the zoospore production. The population of the zoospores in the solution was counted under a light microscope connected with digital images. Attractant activities of **1** and **2** were also tested by means of the particle method. In another series of experiments, 50 mM **11** (4 μ L) or 100 mM **12** (2 μ L, and additionally with 2 μ L pure DMSO) was added to 2 mL of the zoospore suspension to a final concentration of 100 μ M. In the corresponding control, 4 μ L pure DMSO was added instead. Using these zoospore suspensions treated with the adenylate cyclase activator or the inhibitor, we monitored responses of the zoospores toward **1** and **2** on the particles. Also, the numbers of the swimming zoospores that emerged in the solution were captured as photo images under a light microscope (Olympus IX70, Tokyo, Japan) equipped with a high sensitivity cooled CCD color camera (Keyence VB-7010, Osaka, Japan) and counted in a 0.67×0.67 mm² area to be compared with the control.

Results and Discussion

1. Cross-competition assay between flavonoid-type and feruloyl amide-type chemoattractants

When an aqueous solution of cochliophilin A (**1**, 1×10^{-6} M) was used as the solution to allow zoospore production, Chromosorb W/AW carrier particles soaked in a 1×10^{-6} M solution of compound **1** in EtOAc did not attract any zoospores (Table 1). This complete absence of the usual attractant activity of **1** is due to the masking effect by the higher background level of **1** on the concentration gradient of **1** dispersed from the carrier particles. Takayama *et al.* demonstrated that a typically sized porous carrier particle (100 μ m in diameter) can keep 4 nL of the solution on average,¹⁶⁾ therefore, 4 fmol of the chemical substance is carried on particles soaked in a 1×10^{-6} M solution of **1** or **2**. Compound **1** coated on the surface of the porous diatomite particles affected the zoospores for several hours in a hemisphere of *ca.* 1000–1500 μ m diameter around each particle. Considering the volume of the hemispherical space affected by compound **1** as being 2.1 to 7.0 μ L, immediate and uniform dispersal of compound **1** from the particle can be calculated as giving at most a 0.5×10^{-9} to 0.2×10^{-10} M concentration within the hemisphere. In practice, a less hydrophilic compound **1** would be released gradually from the particle to give a concentration gradient of **1**. Therefore, it is concluded that the aqueous solution of **1** in the background contained a 500- to 2000-fold higher concentration of **1** than the hemisphere around the particle. As expected, this background level of **1** is high enough to hide the concentration gradient released from the particles. Compound **2** in the aqueous solution also competed with the attractant activity of compound **2** that was carried on the particle (Table 2).

In the aqueous background solution of **1** (1×10^{-6} M), particles holding 40 fmol of chemoattractant **2** showed clear attractant activity upon applying a 1×10^{-5} M solution of **2**, however, showed clear attractant activity. Considering the concentration gradient of the dispersed **2** from the porous particles, at least a 100-fold higher concentration of **1** in the background indi-

Table 1. Effect of cochliophilin A (**1**), *N*-(*E*)-feruloyl-4-*O*-methyldopamine (**2**), and agonistic compounds in aqueous solution of *Aphanomyces cochlioides* zoospores suspension on **1** carried on the particles

Aqueous solution in background (1×10^{-6})	Concentration of 1 -solution (M) applied to carrier particles			
	1×10^{-5}	1×10^{-6}	1×10^{-7}	1×10^{-8}
(1)	—	—	—	—
2	nt	++	++	++
3	—	—	—	—
4	—	—	nt	—
5	nt	—	nt	—
6	nt	nt	nt	++
7	nt	nt	nt	++

As the responses of *A. cochlioides* zoospores toward a flavonoid-type naturally occurring chemoattractant **1** are dose-dependent, higher concentrations of **1** than the minimum concentration at which the zoospores are actively attracted (++) were not tested (see **6** and **7**). For the attraction of the zoospores, responses of the zoospores were classified into three grades, ++ (highly active), + (active), and — (inactive). nt, not tested. All the responses were observed within 10 min.

Table 2. Effect of cochliophilin A (**1**), *N*-(*E*)-feruloyl-4-*O*-methyldopamine (**2**), and agonistic compounds in aqueous solution of *Aphanomyces cochlioides* zoospores suspension on **2** carried on the particles

Aqueous solution in background (1×10^{-6})	Concentration of 2 -solution (M) applied to carrier particles			
	1×10^{-5}	1×10^{-6}	1×10^{-7}	1×10^{-8}
1	nt	++	—	—
(2)	—	—	—	—
3	nt	++	—	—
4	nt	++	—	—
5	++	++	—	—
6	++	++	—	—
7	nt	++	—	—

The responses of *A. cochlioides* zoospores toward a feruloyl amide-type naturally occurring chemoattractant **2** are dose-dependent. As the zoospores are actively attracted (++) at certain concentration of **2**, higher concentrations than that were sometimes skipped (see **1**, **3**, **4**, and **7**). Responses of the zoospores were classified into ++ (highly active), + (active), and — (inactive). nt, not tested. All the responses were observed within 10 min.

cates that the hypothetical receptor for feruloyl amide **2** does not recognize flavonoid-type chemoattractant **1**. Similarly, *A. cochlioides* zoospores in an aqueous solution of **2** (1×10^{-6} M) were clearly attracted to particles carrying 4 fmol of chemoattractant **1**, although an excessive concentration of compound **2** was present in the background. These observations led to the tentative conclusion that the presumed receptors for sensing chemoattractants **1** and **2** are different.

Compounds **3**, **4** and **5**, all of which have a flavonoid skeleton with a non-substituted B-ring, are agonistic and competitive with **1** in cross-competition assays toward **1** and **2**. As shown in Table 1, the zoospores were all inactive toward the carrier particles when applying 1×10^{-4} M **1** to any of the 1×10^{-6} M aqueous solutions of agonistic flavonoid compounds that showed relatively high attractant activities toward *A. cochlioides* zoospores,¹⁵⁾ while the zoospores were clearly attracted to compound **2** (Table 2). Therefore, these flavonoid-type agonists likely share the flavonoid sensor with **1**.²³⁾ Conversely, aqueous solutions of compounds **6** and **7**, which are structurally analogous to **2** and showed a clear attractant activity toward *A. cochlioides* zoospores, did not compete with attractant activities of **1** and **2** at all. Therefore, **6** and **7** had no competitiveness with **2**. Thus, the hypothetical feruloyl amide receptor seems to selectively recognize **2** among structurally similar compounds, unlike the flavonoid receptor for **1**. Considering the zoospores' distribution in the plant kingdom and their chemical properties and responses, it is most likely that compound **1** is the primary principle for host recognition because of the *A. cochlioides* zoospores' highly sensitive chemotactic activity toward it. Conversely, compound **2** may mediate infection of *A. cochlioides* on the surface of living host roots due to accelerated germination of the encysted cells aggregated after the attraction with **2**.

2. Effects of some purine derivatives and chemical reagents associated with cAMP activation or inactivation on responses of *A. cochlioides* zoospores toward **1** and **2**

Flavonoids, such as wogonin (5,7-dihydroxy-8-methoxyflavone), are often characterized as purine agonists or antagonists.^{24–26)}

The effects of three representative purine derivatives, adenine (**8**), guanine (**9**), and NADP⁺ (**10**), on behavior of *A. cochlioides* zoospores were examined, with each derivative present at 1×10^{-6} M in the zoospore suspension. In particular, it was tested whether the zoospores could respond to the natural attractants **1** and **2** followed by cell aggregation within 1 hr in each solution. In an aqueous 1×10^{-6} M solution of **8**, both attraction and cell aggregation of the zoospores toward **1** and **2** were inactive (—), as shown in Table 3. In the solution of **8**, production of *A. cochlioides* zoospores was obviously reduced. This result indicates that the low population density of the swimming zoospores would be part of the reason why zoospores were rarely attracted by **1** and **2** in a solution of **8**. Indeed, when a 1×10^{-4} M solution of **1** in EtOAc was applied to the particles, the particles coated with **1** attracted zoospores in the suspension and allowed the cells to form plaque-like cell aggregates (data not shown).

In contrast, aqueous solutions of guanine (**9**) and NADP⁺ (**10**) had relatively high densities of swimming zoospores. In spite of such a high density of the zoospores, compound **2** applied to the particles as a 1×10^{-4} M solution in EtOAc was uniquely inactive (—) in an aqueous solution of **10**, leading to neither attraction nor aggregation of the cells. Even after several hours, zoospores did not form any cell aggregates around the particles (Table 3). Conversely, the zoospores in an aqueous solution of **10** displayed active chemotaxis toward compound **1** and finally formed thick cell aggregates. This clear difference in zoospore behavior and responses toward **1** and **2** in an aqueous solution of **10** also supports our tentative conclusion that the receptors of **1** and **2** are different, and these distinguishable sensing systems respectively play an important role in the events of host recognition and consequent infection of root tissues of the host plants.

Islam *et al.*²⁷⁾ suggested that an important intracellular signaling cascade involved in the host-recognition system is likely to be mediated by cAMP, simply because treatment of the swimming zoospores with a G-protein activator, mastoparan,²⁸⁾ led to their encystment followed by spore germination. Although it is simplistic thinking, we examined the effects of the adenylate cyclase activator forskolin (**11**)¹⁸⁾ and the adenylate cyclase

Table 3. Effect of purine derivatives **8**, **9**, and **10** on response of *Aphanomyces cochlioides* zoospores toward high concentrations of chemoattractants **1** and **2** on particles

Aqueous solution in background (1×10^{-6})	Solution of chemoattractant applied to carrier particles			
	1 (1×10^{-5} M)		2 (1×10^{-4} M)	
	Attraction (5 min)	Aggregation (1 hr)	Attraction (5 min)	Aggregation (1 hr)
Adenine 8 ^a	—	—	—	—
Guanine 9	+	++	+	+
NADP ⁺ 10	+	+	—	—
Control	++	++	++	++

All the zoospore attraction was tested by particle method, in which natural attractants **1** and **2** were applied as EtOAc solutions of 1×10^{-5} and 1×10^{-4} M respectively. Zoospore attraction to the particle was observed during 5 min while aggregation around the particle at 1 hr. Activity was ranked as ++ (highly active), + (active), and — (inactive). Aqueous solution in control is Milli-Q water only. ^aIn an aqueous 1×10^{-6} M adenine solution for emergence of *A. cochlioides* zoospores, it obviously reduced population density of the swimming zoospores, and it is a part of the reasons why zoospores are inactive toward **1** and **2** in solution of adenine (**8**).

Fig. 4. Zoospore density of *Aphanomyces cochlioides* with different aqueous solutions in the background. Zoospore density was calculated from 2 mL zoospore suspension in each petri dish (35×10 mm) observed under a light scope (Olympus, Tokyo, Japan) at 100× magnification. An aliquot (4 μL) of 50 mM forskolin (**11**) as a DMSO based stock solution was directly added to 2 mL of zoospore suspension newly prepared. In the zoospore suspension containing 100 μM **11**, a cAMP activator, numbers of swimming zoospores captured as digital images were counted as black dots (A). The control is without **11** (B). Both of the zoospore suspensions treated with and without **11** were prepared in triplicated, and each zoospore suspension observed under the microscope was displayed as four different views randomly selected. Therefore, number of samples (n) in one treatment is 12. The average of the swimming zoospores in the square in each solution was shown in C. Bars, ±SD (n=12), * $p < 0.001$, Student's *t*-test.

inhibitor 9-(2-tetrahydrofuryl)adenine (SQ22536, **12**)¹⁹⁾ on the behavior and responses of *A. cochlioides* zoospores. Due to a literature on a dose–response study of forskolin,²¹⁾ a 100 μM aqueous solution of **11** was used to examine zoospore production. The results showed the population density of the swimming zoospores drastically increased, along with acceleration of their motility (Fig. 4). Even in zoospore suspensions in RO water that had been added to 24-well or a 12-well plates (2 mL each), later addition of **11** to a final concentration of 100 μM led to an obvious increase in the number of swimming zoospores. This incremental effect of **11** on the zoospores is probably due to acceleration of zoospore regeneration from encysted cells due to mechanical stimulation by vortexing⁹⁾ and chemical stimulation *via* incremental levels of endogenous cAMP. A 100 μM solution of **11** also restored the population density of swimming zoospores following encystment in a 1×10^{-6} M aqueous solution of **8**. In contrast, exogenous cAMP at 1×10^{-6} M neither affected motility of the zoospore cells nor increased population density of the swimming zoospores. Furthermore, 100 μM SQ22536, which is a

one-fifth concentration of that reported in an earlier study,²²⁾ repressed the population size of the swimming zoospores to nearly zero (data not shown).

3. Further discussion

The stimulation of zoospore motility by **11** suggested an important role of intracellular cAMP as a key signal transducer for kliekinetic regulation of the zoospores. Indeed, zoospores treated with a 100 μM aqueous solution of **11** maintained active chemotaxis to **1** and **2** without any reduction in the population density. The zoospores activated by **11** often swam across the whole view (4×3 mm²) visible under a 40× light microscope. In contrast, the zoospores exposed to both **11** and 1×10^{-6} M **1** tended to stay in a relatively narrow area (*ca.* a circle with a radius of 0.3 mm) compared to those treated only with a 100 μM aqueous solution of **11**.

If **1** and **2** shared a single sensory receptor, an approximately 1,000-fold higher level of one attractant in the background would have masked the concentration gradient of another at-

tractant carried on the particle. However, the result of our bioassay showed that the weak concentration gradient of one attractant is maintained around the particle, despite the presence of such an excessive concentration of another attractant in the surrounding aqueous solution. Thus, the cross-competition assay among naturally occurring zoospore attractants **1** and **2** and their analogous compounds clearly indicated that **1** and **2** are non-competitive, suggesting the presence of two distinct chemosensory receptors for **1** and **2**. The totally different sensitivity of the zoospores toward **1** and **2** in the presence of NADP⁺ (**10**) also supported this speculation. Thus, the hypothetical receptor for **1**, which is highly sensitive but accepting of a wide range of structurally related analogs, is likely far different from the receptor for **2**, which has relatively lower sensitivity but a strict chemical structure requirement. Our further study of the flavonoid receptor that links the chemotaxis of the zoospores to **1** will be reported elsewhere.

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