Nicotinamide and Structurally Related Compounds Show Halting Activity against Zoospores of the Phytopathogenic Fungus *Aphanomyces cochlioides*

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In a survey of plant secondary metabolites regulating the behavior of phytopathogenic *Aphanomyces cochlioides* zoospores, we found that leaf extracts of *Amaranthus gangeticus* and cotyledon extracts of pea (*Pisum sativum*) remarkably halted the motility of zoospores. Bioassay-directed fractionation of *A. gangeticus* and pea constituents revealed that the halting activity was dependent on a single chemical factor (halting factor). The active principle was identified as nicotinamide (**1**) by comparing its biological activity and spectroscopic properties with those of the authentic compound. Nicotinamide (**1**) showed potent halting activity toward the zoospores of *A. cochlioides* and *A. euteiches*, but it exhibited very less activity against other Oomycetes, *Pythium aphanidermatum* and *Phytophthora infestans* zoospores. Interestingly, the zoospores halted by nicotinamide (**1**) encysted within 10–15 min and then the resulting cystospores regenerated zoospores instead of germination. Nicotinamide (**1**) and related compounds were subjected to the halting activity bioassay to elucidate the structure-activity relationships. These bioassays revealed that part structures of (A) the aromatic ring containing at least one nitrogen atom, (B) carbonyl-like group adjacent to the aromatic ring and (C) hydrogen atoms on the amide group are responsible for the strong activity. So far, this is the first report of halting activity of nicotinamide (**1**) against fungal zoospores.

Introduction

Aphanomyces cochlioides Drechsler (Saprolegniaceae) is a soil-borne phytopathogenic fungus which is responsible for a root rot disease of spinach (*Spinacia oleracea* L.) (Ui and Nakamura, 1963) and a damping-off disease of sugar beet (*Beta vulgaris* var. *rapa* Dum.) (Drechsler, 1928). The fungus also infects some other species of Chenopodiaceae and Amaranthaceae (Ui and Nakamura, 1963). The biflagellate 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 host specific constituents in the root exudates attract zoospores to the host plant where they aggregate as a hemispheric mass on the root surface and encyst before germinate and penetrate into the host tissues. Zoospores of *A. cochlioides* aggregate at the host root by chemotaxis and then undergo a sequence of physiological changes leading to the infection (Islam *et al.*, 2001). We identified cochliophilin A (5-hydroxy-6,7-methylenedioxyflavone) as a host-specific attractant of the *A. cochlioides* zoospores from the roots of spinach (Horio *et al.*, 1992). The gradient of cochliophilin A induces encystment of the zoospores, followed by germination of the cystospores. The differentiation of zoospores by cochliophilin A was supposed to be initiated by stimulation of a reputed G-protein-coupled receptor which activates a signal transduction system including phosphoinositoids and Ca^{2+} (Islam and Tahara, 2001).

During a survey of plant secondary metabolites regulating the behavior of *A. cochlioides* zoospores, we observed that the crude extracts of *Amaranthus gangeticus* and pea seedlings strongly inhibited the motility of zoospores. This prompted us to isolate the zoospore halting factor(s) from *A. gangeticus* leaves and garden pea seedlings by detailed bioassay-guided fractionation. Here we

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report the isolation and identification of the halting factor from these plants, and discuss its biological activity toward fungal zoospores.

Materials and Methods

General

The silica gel 60 (mesh 100–200 μm) was used for column chromatography while the purity of samples was checked on Merck silica gel 60 F_{254}, 0.25 mm-thick TLC plates. The spots were viewed under 254 or 365 nm UV light and by spraying with 5% H_2SO_4 in EtOH. The Whatman 3MM3 was used for preparative PC. The Merck DIOL $F_{254}S$, 0.2 mm-thick HPTLC plates were used for preparative TLC. The mass spectra were recorded on a JEOL JMS-AX500 and, a JEOL JNM-EX 270 for recording ^1H NMR spectra.

Production of zoospores and bioassay

The fungi including *A. cochlioides* (AC-5) which was isolated from the soil of a sugar beet field, were cultured for 5–6 days on a corn meal agar (Difco) plate at 20 °C. The production of zoospore and the "particle bioassay" were carried out as previously reported (Horio *et al.*, 1992; Mizutani *et al.*, 1998; Islam and Tahara, 2001). Briefly, one drop of test solution (a 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. It is estimated that each particle holds the amount of compounds equivalent to about 4 nl of the test solution (Takayama, 1999). One to two of these particles were carefully dropped into 2 ml of zoospore suspension (*ca.* 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 30 min after addition of the particle(s). Control particles were treated with solvent alone.

When the particle contains enough amount of the halting factor, the zoospores around the particle stop swimming in 1 min and encyst to give a high number of cystospores around the particle, whilst the zoospores were continuingly swimming for several hours around a control particle.

The "homogenous solution method" was also carried out to measure the halting activities of compounds **1**–**10** in the zoospore suspension where each test compound was homogeneously dissolved. To 2 ml of the zoospore suspension into a small Petri dish (3 cm i. d.), 3 μl of the DMSO solution of each test compound was added, and quickly but gently mixed well to give a homogenous solution. The behavior of zoospores was observed microscopically up to 20–30 min after addition of the solution. A control was run simultaneously using 3 μl of DMSO in place of the test solution.

Halting activity in the homogeneous method was quantified as follows.

Halting index (HI) = $100 \times$ (B-C) / (A-C)

A: Number of encysted zoospores in 1×10^{-5} M nicotinamide solution where swimming zoospores disappeared in a few min and settled themselves on the bottom of the Petri dish.

B: Number of encysted zoospores in the test solution.

C: Number of encysted zoospores in the control solution (the zoospore suspension and DMSO).

The numbers of A, B and C were counted at least 5 microscopic fields of each Petri dish and averaged.

It is possible to combine the particle method with the homogeneous solution method when we wished to know the interrelation of two compounds, for example agonistic and antagonistic ones. One of them is supplied by the particle method into the zoospore suspension homogeneously dissolved with the other compound.

Plant materials and extraction

Amaranthus gangeticus (cv. Altapati) were grown in the experimental farm of Hokkaido University, from May to August 2000. The whole plants were collected at their flowering stage and separated into leaves, stems and roots, and allowed to dry at room temperature in shade. The air dried leaves (1.67 kg) were ground by an electric mill, and repeatedly extracted with 50% acetone. The combined acetone extract from 1.67 kg of the dried leaves was concentrated, and the residue was partitioned with *n*-hexane, diethyl ether, EtOAc and MeOH to yield *n*-hexane (0.71 g), diethyl ether (2.1 g), EtOAc (10.0 g) and MeOH (167.0 g) fractions, respectively.

The cotyledons of pea (*Pisum sativum*) were extracted with hot water and the extract was concentrated to dryness. The residue was extracted successively with *n*-hexane and acetone. The acetone soluble constituents in water were subsequently partitioned with diethyl ether and *n*-BuOH to yield diethyl ether and *n*-BuOH fractions respectively.

Chemicals

The following commercially available chemicals were of the highest purity and unless otherwise stated were used without further purification: nicotinamide (**1**), pyrazinamide (**2**), thionicotinamide (**3**), nicotinamide adenine dinucleotide (NAD) (oxidized form) (**4**), nicotinamide adenine dinucleotide phosphate (NADP) (oxidized form) (**5**), β-nicotinamide mononucleotide (oxidized form) (**6**), isonicotinamide (**7**), 2,6-dichloroisonicotinamide (**8**), malonic acid (**9**), malic acid (**10**), phenol (**11**), *ortho-tert*-butylphenol (**12**), salicylic acid (**13**), 2-pyridinecarboxamide (picolinamide) (**14**), benzamide (**15**), anthranilamide (**16**), nipecotinamide (**17**), 3-(aminomethyl)pyridine (**21**), *N*-methylnicotinamide (**22**), *N,N*-dimethylnicotinamide (**23**), *N,N*-diethylnicotinamide (**24**), nicotinanilide (**25**), nicotinic acid (**26**), pyridine (**27**), 3-aminopyridine (**28**), nicotinitrile (**29**), 3-pyridylacetonitrile (**30**), *trans*-3-(3-pyridyl)acrylic acid (**31**), (−)-nicotin (**32**), 3-pyridylacetamide (**33**), anthranilic acid (**34**), 5-acetylsalicylamide (**35**), acetylsalicylic acid (**36**), acetylsalicylic acid methyl ester (**37**), 2-pyridylacetonitrile (**38**), 3,4-pyridinedicarboximide (**39**), benzylamine (**40**), 2,4-di-*tert*-butylphenol (**41**), 4-chloro-2-methylphenol (**42**), resorcinol (**43**), salicylaldehyde (**44**), D-biotin (**45**), L-ascorbic acid (**46**), pyridoxamine (**47**), pyridoxine (**48**), pyridoxic acid (**49**), adenosine 5′-triphosphate (ATP) (**50**), and nicotinamide adenine dinucleotide (NADH) (reduced form) (**51**).

Preparation of pyrrole-3-carboxamide (**18**)*, furan-3-carboxamide* (**19**)*, and thiophene-3-carboxamide* (**20**)

To a solution of pyrrole-3-carboxylic acid (50.0 mg), furan-3-carboxylic acid (51.2 mg), or thiophene-3-carboxylic acid (50.0 mg) in acetonitrile (2 ml) was added *N,N′*-carbonyldiimidazole (81.5 mg) and each mixture was stirred for 1 h at room temperature. Sodium amide (~10 mg) was added to the mixture, which was further stirred at room temperature for 30 min. The solvent was reduced to give a solid which was purified by silica gel column chromatography using toluene-acetone = 10:1. Thus, pyrrole-3-carboxamide (**18**) (25.3 mg) (51% yield) (Sycheva *et al.*, 1962), furan-3-carboxamide (**19**) (24.0 mg) (48% yield) (Reichstein and Zschokke, 1932), thiophene-3-carboxamide (**20**) (24.8 mg) (50% yield) (Inamori *et al.*, 1994) were prepared.

Preparation of 3-pyridylacetamide (**33**)

A solution of 3-pyridylacetic acid hydrochloride (200 mg) in a mixture of concentrated H_2SO_4 (1 ml) and absolute EtOH (5 ml) was stirred for 3 h at 95 °C and poured into crushed ice (50 g). An excess amount of conc. ammonia water was added to the solution and the resulting alkaline solution was extracted with EtOAc. The EtOAc extract was concentrated to yield a solid, to which was added 3 ml of conc. ammonia water and followed by stirring for 3 h at room temperature. The product was purified by silica gel column chromatography using $CHCl_3$-MeOH = 5:1 (v/v) to give 71.9 mg of 3-pyridylacetamide (**33**) in a 46% yield (Burger and Walter, 1950).

Results and Discussion

Isolation and identification of the halting factor against zoospores

As shown in Fig. 1, the acetone extracts of *Amaranthus gangeticus* leaves were divided into *n*-hexane, diethyl ether, EtOAc and MeOH soluble fractions. The MeOH soluble constituents were subjected to silica gel column chromatography, and the halting factor was eluted with $CHCl_3$-MeOH = 9:1. The active fractions were concentrated and rechromatographed over a silica gel column with a solvent system of EtOAc-MeOH = 10:1. The active principle (0.4 mg, highly pure, but in poor yield) was finally isolated by preparative TLC using DIOL F_{254} s plates (chemically modified silica gel coated, Merck) in $CHCl_3$-MeOH = 10:1 v/v. The isolation yield was calculated to be 1.3 mg from 1 kg of air-dried leaves of *A. gangeticus*, however according to the biological activity and the amount of Frs. 17–29 in Fig. 1, the content was estimated to be more than 40 mg/kg or so.

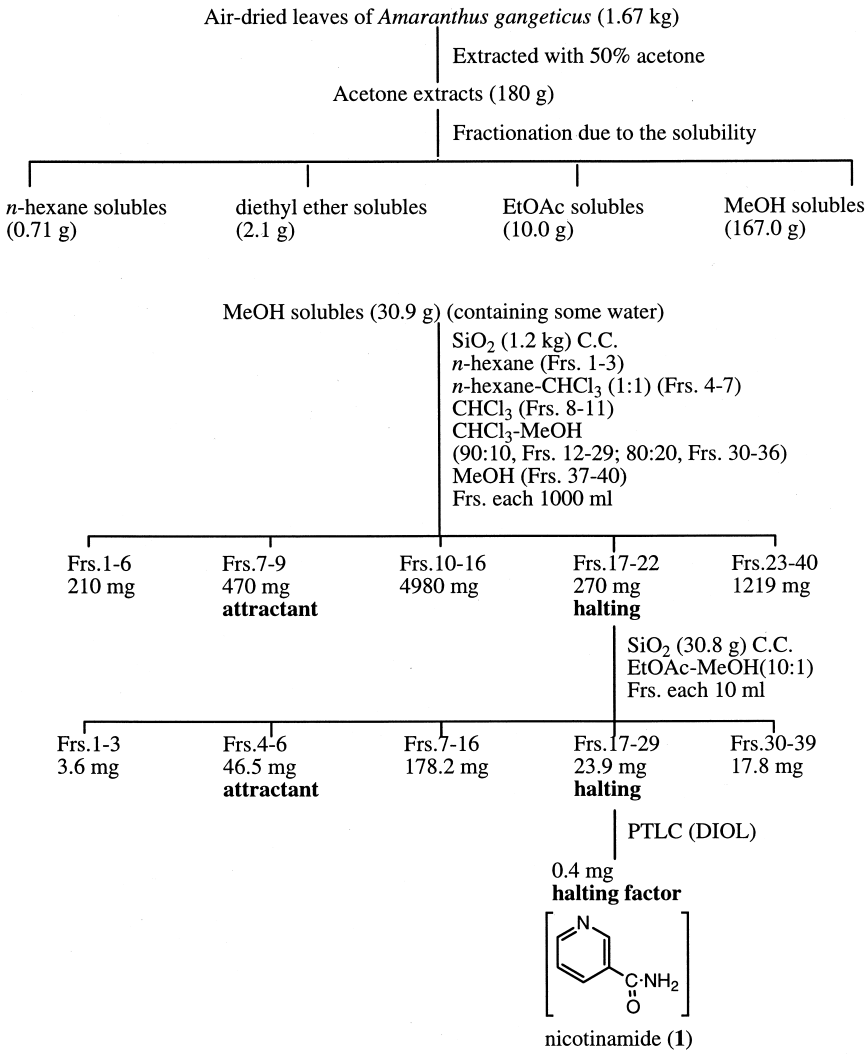


Fig. 1. Isolation procedure for the zoospore halting factor in *Amaranthus gangeticus* leaves. SiO_2 C. C.: silica gel column chromatography.

The HR-EI-MS analysis of the isolate revealed the molecular formula to be $C_6H_6N_2O$ (*m/z*: 122.0473). The EI-MS (*m/z*) (relative int.) gave fragments at 122 (M^+ 100), 106 (51), 78 (56), 51 (30) and 44 (13). The ^1H NMR spectrometry revealed the presence of four aromatic protons [δ 7.53 (1H, ddd, J = 8.0, 5.0, 0.8 Hz), δ 8.27 (1H, ddd, J = 8.0, 1.6, 1.6 Hz), δ 8.68 (1H, ddd, J = 5.0, 1.6, 1.6 Hz) and δ 9.01 (1H, br. t-like, J = 1.6 Hz)] which resembled well with four protons on 3-substituted pyridine. These values were indeed in fair agreement with those of reported nicotinamide (Yamashita *et al.*, 1989). The ^1H NMR and EI-MS spectra of authentic nicotinamide were identical to those of the isolate. Thus the halting factor of *A. gangeticus* has been unambiguously identified as nicotinamide (**1**). Furthermore, the halting activity of authentic nicotinamide (**1**) was also equivalent to that of the *A. gangeticus* isolate (Fig. 2).

The *n*-BuOH extracts of pea cotyledons was subjected to preparative PC using Whatman 3MM3 in *n*-BuOH-EtOH-H_2O = 12:3:5 to isolate an active principle. The physicochemical properties and biological activity of the isolate were com-

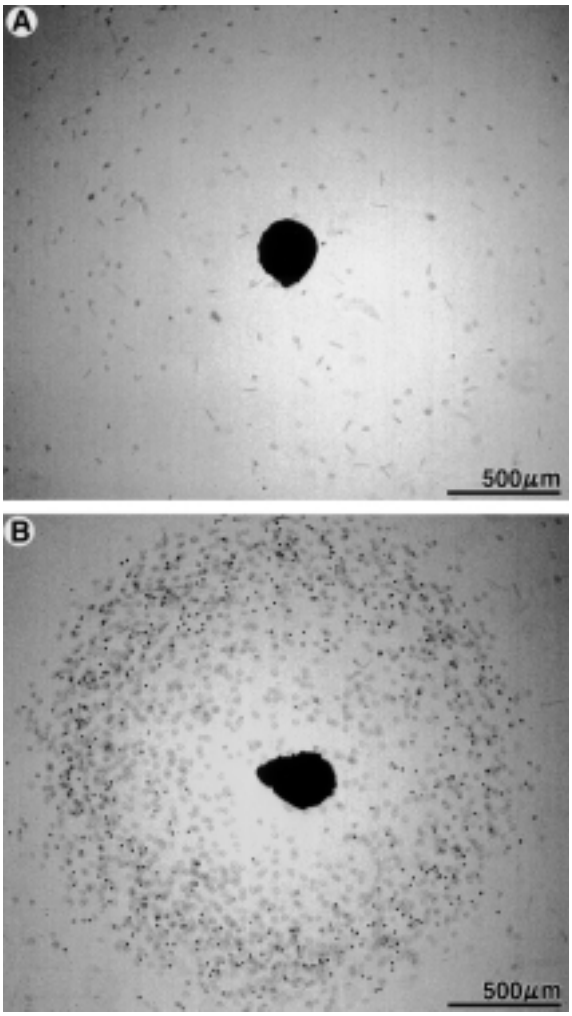


Fig. 2A-B. Photomicrographs of zoospores of *Aphanomyces cochlioides* (A) control, and (B) after exposure to nicotinamide (**1**) released from Chromosorb W AW (150–200 μm) particle coated with 8.2×10^{-4} M solution, an unusually high concentration to show the effect remarkably. Photographs were taken through a microscope with a 40× magnification and an exposure time of 0.3 s. Both tiny black spots and faint circles (a little distant from the microscopic focus) in (A): halted zoospores. Lines in (A) and in the area remote from the particle (B): traces of swimming zoospores.

pletely identical to those of *Amaranthus* and authentic nicotinamide (**1**).

Halting activity of nicotinamide and related compounds

The motility inhibiting activity of nicotinamide (**1**) has been first observed against the zoospores of *A. cochlioides*. However, a further test using some kinds of zoospores revealed that both *A. cochlioides* and *A. euteiches* showed high sensitivity to **1** (all zoospores halted instantly at 8.2×10^{-7} M), while the zoospores of *Pythium aphanidermatum* 71–81, *P. aphanidermatum* 72–22, and *Phytophthora infestans* Pio-761-s exhibited a weak response to **1** at 4.1×10^{-4} M, 1.5×10^{-5} M, and 4.1×10^{-1} M, respectively. We found that the motility of zoospores treated with nicotinamide and its related compounds were immediately inhibited within 1 min, followed by encystment within 10–15 min. Interestingly, almost all of the encysted zoospores regenerated zoospores in further 5–15 min instead of yielding mature cystospores and germinating.

The zoospores of *A. cochlioides* are attracted to the host roots by chemotaxis which results in a hemispheric mass of the zoospores on the surface of roots, where they encyst, germinate and penetrate into the root tissues (Islam *et al.*, 2001). This sequencial phenomenon is released by the host-specific signal, cochliophilin A from the roots of spinach. However the phenomenon caused by nicotinamide resembled the fate of vortex-induced cystospores or cysts incubated with Ca^{2+} flux inhibitors. It has been established that transmembrane Ca^{2+} fluxes are essential for encystment and germination of all Oomycetes zoospores (Connolly *et al.*, 1999; Warburton and Deacon, 1998).

We previously reported that interaction of two chemically different factors isolated from *Portulaca oleracea* could halt *A. cochlioides* zoospores. One of these compounds was *N-trans*-feruloytyramine, which by itself was active as a zoospore stimulant, and the other one was an acidic compound, 1-linoleoyl-2-lysophosphatidic acid monomethyl ester, which had zoospore-repellent activity. When Chromosorob 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 the 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 (Mizutani *et al.*, 1998).

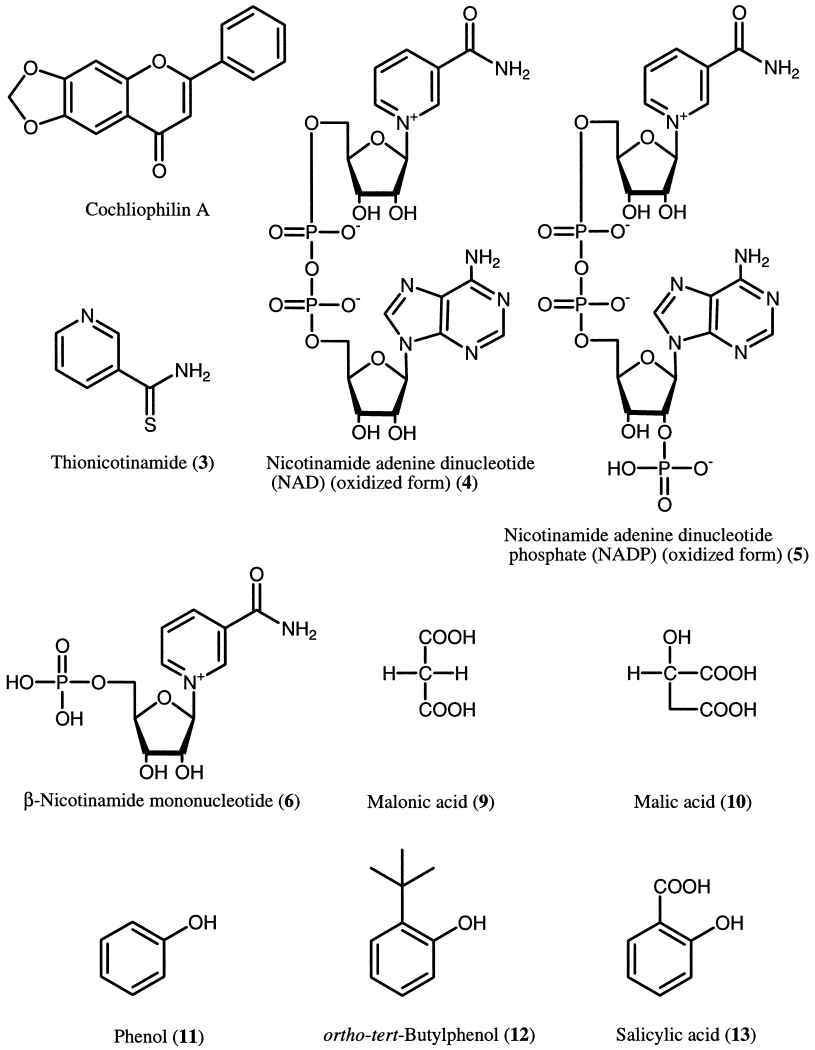


Fig. 3. Some physiologically active compounds toward *Aphanomyces cochlioides* zoospores.

Structure-activity relationships

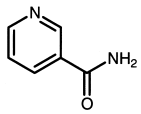
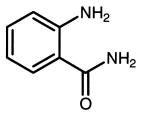
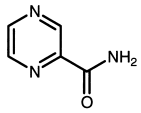
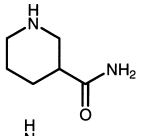
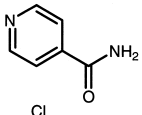
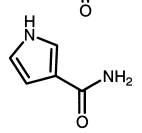
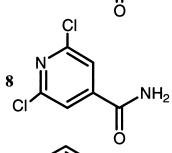
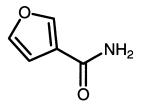
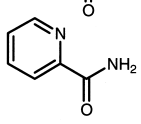
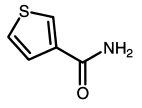
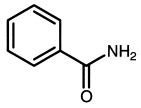
Commercially available or chemically prepared nicotinamide (**1**) and its related compounds **2**–**51** were subjected to the halting activity bioassay toward the zoospores of *A. cochlioides* and the results are summarized in Tables I, II and III. The highest activity was recorded in pyrazinamide (**2**) (particle method 8.1×10^{-6} M, homogenous solution method 8.1×10^{-9} M), thionicotinamide (**3**) (particle method 7.2×10^{-6} M, homogenous solution method 7.2×10^{-9} M), followed by nicotinamide (**1**) (particle method 8.2×10^{-5} M, homogenous solution method 8.2×10^{-8} M). Nicotinamide adenine dinucleotide (NAD), oxidized form (**4**), nicotinamide adenine dinucleotide phosphate (NADP), oxidized form (**5**), β-nicotinamide mononucleotide, oxidized form (**6**) showed halting activity at *ca.* 10^{-4} M in particle method, and *ca.* 10^{-7} M in homogenous solution method, while isonicotinamide (**7**), 2,6-dichloroisonicotinamide (**8**), malonic acid (**9**), malic acid (**10**), phenol (**11**), *ortho-tert*-butylphenol (**12**) and salicylic acid (**13**) displayed clear halting activity at *ca.* 10^{-3} M in particle method, and *ca.* 10^{-6} M in homogenous solution method. However halted zoospores by the

Table I. Zoospore halting activity of nicotinamide and related compounds.

Test compound	Active concentration [M]	
	Particle method[a]	Homogenus solution method[b]
Nicotinamide (**1**)	8.2×10^{-5}	8.2×10^{-8}
Pyrazinamide (**2**)	8.1×10^{-6}	8.1×10^{-9}
Thionicotinamide (**3**)	7.2×10^{-6}	7.2×10^{-9}
Nicotinamide adenine dinucleotide (NAD) (oxidized form) (**4**)	1.5×10^{-4}	1.5×10^{-7}
Nicotinamide adenine dinucleotide phosphate (NADP) (oxidized form) (**5**)	1.3×10^{-4}	1.3×10^{-7}
β-Nicotinamide mononucleotide (**6**)	3.0×10^{-4}	3.0×10^{-7}
Isonicotinamide (**7**)	8.2×10^{-3}	8.2×10^{-6}
2,6-Dichloroisonicotinamide (**8**)	5.2×10^{-6}	5.2×10^{-6}
Malonic acid (**9**)	9.6×10^{-3} [c]	9.6×10^{-6}
Malic acid (**10**)	7.5×10^{-3} [c]	7.5×10^{-6}
Phenol (**11**)	1.1×10^{-3} [c]	NT [d]
ortho-tert-Butylphenol (**12**)	6.7×10^{-3} [c]	NT [d]
Salicylic acid (**13**)	7.2×10^{-3} [c]	NT [d]

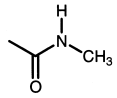
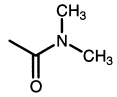
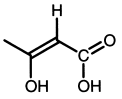
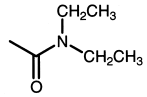
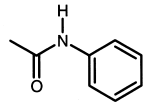
[a] When Chromosorb W AW particles coated with each test solution at the shown concentration were added to a suspension of zoospores, the zoospores around the particles were fully inhibited to swim.
[b] Homogenus solution method: the clear activity (HI > 10, see the text) was observed at the shown concentration, whilst the activity disappeared at the one-tenth concentration. The lowest active concentration was evaluated due to the halting index bigger than 10.
[c] Although these compounds (**9–13**) inhibited the zoospore motility, the halted zoospores could not changed into usual cystospores and failed into the regeneration of the zoospores. [d]NT: not tested.

Table II. Halting activity of amides differing in the ring structure[a].

Compound	Halting activity [M]	Compound	Halting activity [M]
1 	8.2×10^{-5}	**16** 	na
2 	8.1×10^{-6}	**17** 	na
7 	8.2×10^{-3}	**18** 	na
8 	5.2×10^{-3}	**19** 	na
14 	na[b]	**20** 	na
15 	na		

[a] Particle method, see the footnote to Table I.
[b] na: not active at 1000 ppm (*ca.* 10^{-2} M).

Table III. Halting activity of compounds differing in the side substituents[a].

Substituent (R)	Halting activity [M]	Substituent (R)	Halting activity [M]
1 	8.2×10^{-5}	**27**	na
3 	7.2×10^{-6}	**28** 	na
21 	na[b]	**29**	na
22 	na	**30** 	na
23 	na	**31** 	na
24 	na	**32** 	na
25 	na	**33** 	na
26 	na		

[a] Particle method, see the footnote to Table I.
[b] na: not active at 1000 ppm (*ca.* 10^{-2} M).

latter compounds, three acids (**9**, **10**, **13**) and two phenols (**11**, **12**) remained unchanged up to 60 min and they did not regenerate the new generation of zoospores suggesting that the quality in biological activity of **9**–**13** may be different from that of nicotinamide (**1**). Compounds **34**–**51** shown in the section of Methods and Materials were inactive at *ca.* 10^{-2} M by the particle method. In homogeneous solution method, the halting activities were proportional to the results of the particle method (Table I). We also tried to find antagonists against nicotinamide, however each one of inactive compounds **14**–**51** in the zoospore suspension at *ca.* 10^{-5} M did not show any antagonistic activity against nicotinamide administered by particle method (8.2 x10^{-5} M).

The structure-activity relationships among nicotinamide derivatives and structurally related compounds are summarized as follows. An aromatic ring contained two nitrogen atoms at 1, 4 positions seemed to increase the halting activity slightly, but other ring (compounds **14**–**20**) exhibited no activity (Table II). Thiocarbonyl group showed the activity, but 3-(aminomethyl)pyridine (**21**) was inactive. A comparison of halting activity of **1** with that of **22**–**25** revealed the significant contribution of the hydrogen atoms of an amide group to the intensity of activity (Table III). The structural requirements for the zoospore halting activity are quite less versatile. These results show that (A) the aromatic ring containing nitrogen atoms, (B) a carbonyl-like group (carbonyl or thiocarbonyl)

adjacent to an aromatic ring and (C) hydrogen atoms at the side-chain amide group are quite significant to afford strong halting activity toward the *A. cochlioides* zoospores.

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Burger A. and Walter C. R., Jr. (1950), Some α-substituted β-pyridylethylamines. J. Am. Chem. Soc. **72**, 1988–1990.

Connolly M. S., Williams N., Heckman C. A. and Morris P. F. (1999), Soybean isoflavones trigger a calcium influx in *Phytophthora sojae*. Fungal Genet. Biol. **28**, 6–11.

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

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.

Inamori Y., Muro C., Osaka K., Funakoshi Y., Usami Y., Tsujibo H. and Numata A. (1994), Inhibitory activities of 3-thiophenecarboxylic acid and related compounds on plant growth. Biosci. Biotechnol. Biochem. **58**, 1336–1337.

Islam M. T., Ito T. and Tahara S. (2001), Morphological studies on zoospores of *Aphanomyces cochlioides* and changes during interaction with plant materials. J. Gen. Plant Pathol. **67**, 255–261.

Islam, M. T. and Tahara S. (2001), Chemotaxis of fungal zoospores, with special reference to *Aphanomyces cochlioides*. Biosci. Biotechnol. Biochem. **65**, 1933–1948.

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.

Reichstein V. T. and Zschokke H. (1932), Über Furan-β-carbonsäure. Helv. Chim. Acta **15**, 268–273.

Sycheva T. P., Pankina Z. A. and Shchukina M. N. (1962), Compounds with potential antituberculous activity VII. Thiopyrrolecarboxamides. J. Gen. Chem. USSR **33**, 3585–3589.

Takayama T. (March 1999), Ecochemical studies on chemotaxis of spinach root rot pathogen, *Aphanomyces cochlioides* zoospores. PhD Thesis, Grad. Sch. Agric., Hokkaido University, Sapporo, Japan (in Japanese), pp.27–40.

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).

Warburton A. J. and Deacon J. W. (1998), Transmembrane Ca^{+2} fluxes associated with zoospore encystment and cyst germination by the phytopathogen *Phytophthora parasitica*. Fungal Genet. Biol. **25**, 54–62.

Yamashita Y., Sakata K., Ina H. and Ina K. (1989), Isolation of nicotinamide from *Mallotus* leaves as an attaching repellent against the blue mussel, *Mytilus edulis*. Agric. Biol. Chem. **53**, 3351–3352.