# Methin as a nematode attractant in Allium sativum

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Title:
Methiin as a nematode attractant in *Allium sativum*

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Abstract:

Damage to garlic (*Allium sativum*) caused by nematodes *Ditylenchus destructor* is becoming a serious agricultural hazard, leading to a great loss in garlic production. Once the garlic bulbs are invaded, the pathogenic nematode drastically increases in number along with the rotting of bulbs. It was therefore conceived that nematode attractants are present in the bulbs. Based on this hypothesis, chemical investigations were performed to explore a nematode attractant in *A. sativum* bulbs, which resulted in the identification of methiin (S-methyl-L-cysteine S-oxide) as an attractant. Bioassay and quantification experiments of methiin in extracts of *A. sativum* bulb led to the conclusion that methiin possesses sufficient potential to attract *D. destructor* into *A. sativum* bulbs. Moreover, an activity comparing study of methiin with its analogs showed that the sulfoxide functionality is essential for attractant activity. Moreover, methiin was revealed to attract *Caenorhabditis elegans*. Further investigation of methiin will help to elucidate the neuronal system of *D. destructor*.

Keywords:

*S*-methyl L-cysteine S-oxide, alliinase, attractant activity against nematodes, *Ditylenchus destructor* Throne, *Caenorhabditis elegans*
Introduction

Garlic (*Allium sativum*) is an agricultural staple in Aomori, Japan. In 1984, the *A. sativum* crops were damaged, including the stunting and rotting of bulbs, which led to a great loss in garlic production. A study of Fujimura *et al.* discovered that damage to the bulbs was mainly caused by potato rot nematode also known as *Ditylenchus destructor* Thorne\(^1,2\). This is an internationally quarantined nematode and is known to be a destructive pest in a number of roots and tube crops\(^3-6\). For example, the pathogenic nematode is the second-ranking nematode pest of potatoes after the potato cyst nematode in Europe and North America. In China, *D. destructor* has been one of the three major diseases that restrict the production of sweet potato. It has also been reported that *D. destructor* increases in number during infestation of *A. sativum* bulbs, which is dramatically accompanied with the rotting of bulbs\(^7,8\). The exclusive feature of *A. sativum* bulbs invaded by *D. destructor* was not observed in other crops.

*D. destructor* is positioned at the base of the most economically important plant nematodes, including root-knot and cyst nematodes, on the small subunit (SSU) rRNA tree\(^9\). Usually, reproduction of plant-parasitic nematodes, such as root-knot and cyst nematodes, obligately relies on plants, and they are difficult to manipulate using molecular methods. Interestingly, *D. destructor* can feed on fungi or potatoes easier and has a relatively short life cycle, suggesting its use as a model for biological and biocontrol studies of plant-parasitic nematodes\(^10\). In fact, studies on the genomic analyses of *D. destructor* have been progressing. For example, Zheng *et al.*\(^11\) demonstrated that in *D. destructor*, the core developmental control processes have undergone heavy reduction, whereas most signal transduction pathways have been conserved, as opposed to that of *Caenorhabditis elegans*. Ding *et al.* developed a rapid diagnosis method using loop-mediated isothermal amplification assay based on 28S rRNA sequences\(^12\). Regarding the infection mechanism, Cheng *et al.*\(^13\) determined the relationships...
of *D. destructor* densities in soil, garlic roots and outer skins of garlic bulbs, and damage to bulbs that rot during storage using the real-time PCR method. The results indicated that *D. destructor* densities in roots and outer skins may be a good indicator to estimate nematode damage to garlic bulbs after storage. Peng *et al.*\textsuperscript{14} undertook the approach of sequencing expressed sequence tags (ESTs) derived from a mixed stage cDNA library of *D. destructor*, clarifying that the migratory plant parasitic nematode *D. destructor* secretes similar effectors to those of sedentary plant nematodes. Xu *et al.* reported an attractant effect of sweet potato root exudates\textsuperscript{15}, suggesting that *D. destructor* approaches crops under the guidance of root exudates, while the structure remains unclear.

As described above, the feature of *A. sativum* bulbs invaded by *D. destructor* was exclusive unlike other crops. As the cause, it was hypothesized that nematode attractant(s) may be present in *A. sativum* bulbs, which could act as a new type of pesticide for *A. sativum* production. However, a study on nematode attractant(s) in *A. sativum* bulbs has not been reported, while many studies on *D. destructor* has been known. Thus, following the above hypothesis, we chemically investigated for nematode attractant(s) in *A. sativum* bulbs, which resulted in the identification of methiin (1, S-methyl-L-cysteine S-oxide).

**Experimental section**

**General**

The \textsuperscript{1}H (500 MHz) and \textsuperscript{13}C (125 MHz) NMR spectra were recorded in D\textsubscript{2}O using a Jeol JNM-ECA 500 spectrometer. The remaining proton signal for HDO (\textit{\delta}_{\text{H}} 4.63 ppm) was used as an internal standard in the \textsuperscript{1}H NMR spectra. For the \textsuperscript{13}C NMR spectra, the default offset was employed and not corrected. Electrospray-ionization mass spectra were obtained by a Hitachi NanoFrontier LD spectrometer. The worms were counted using a SSZ-B stereomicroscope of Kyowa Optical Co., Ltd.
The infested bulbs of *A. sativum* were harvested from our experimental field. Other chemicals were purchased from commercial sources and used without further purification.

**Preparation of *A. sativum* extract**

*A. sativum* bulbs (50.0 g) were crushed in 50 mM aqueous CaCl\(_2\) (150 mL), and the mixture was filtered through two pieces of gauze to afford the *A. sativum* juice. The juice was used in the assay in Fig. 1a after adequate dilution.

*A. sativum* bulb powder (12.3 g), prepared from freeze-dried bulbs, was extracted in 50% MeOH containing 1% TFA (600 mL). After sonication and filtration, the obtained solution was concentrated *in vacuo* to afford the alliinase-inactive extract. The extract was used in the assay in Fig. 1b and in quantification experiments of compound \(1\) after adequate dilution. The alliinase-active extract was also prepared in similar way.

**Quantification of methiin using HPLC-ESI/MS**

Both alliinase-active and inactive extract solutions (20 mg DW/mL) described above were subjected to HPLC-ESI/MS, respectively. Quantification experiment of compound \(1\) was performed using HPLC-ESI/MS (column: COSMOSIL® HILIC, 2.0 mm ID × 150 mm, solvent: 70% aqueous MeCN containing 0.1% HCOOH, flow rate: 0.2 mL/min, retention: \(t_R = 3.8\) min; MS mode: positive, detection: \(m/z\) 152.04 [M+H]\(^+\) (C\(_4\)H\(_{10}\)NO\(_3\)S requires 152.0381), ion spray voltage: 3500 V, curtain gas: nitrogen, scan range: 50-2000 V, RF voltage: 500 V, spectrum acquisition: 1.2 s/spectrum, sample injection: 1 \(\mu\)L). From the calibration curve, the linearity between the injected \(1\) and the peak area at \(m/z\) 152.04 was observed. The formula is expressed as giving in equation 1 (\(x\) is concentration (mg/mL) of the injected \(1\), and \(y\) is the peak area at \(m/z\) 152.04; \(R^2 = 0.999\), calculated using Excel2013 software). This method enabled us to quantify 15 pmol (2.3 ng, 2.3 \(\mu\)g/mL) of compound \(1\).
\[ y = 4.36 \times 10^7 x - 2.14 \times 10^4 \] (eq.1)

The peak area of compound 1 in the alliinase-inactive extract solution was 621000, corresponding to 15 μg/mL, while compound 1 was not detected in the alliinase-active extract solution (< 2.3 μmol/mL).

**S-propyl-L-cysteine**

To a suspension of L-cysteine (1.00 g, 8.25 mmol) in ethanol (25 mL) mixed with 20 M of aqueous sodium hydroxide (1.45 mL), propyl bromide (0.83 mL, 9.1 mmol) was added with vigorous stirring at 60°C for 30 min. To this mixture was added acetic acid (1.7 mL), and the mixture was left at 0°C overnight to afford a light yellow solid. The solid was washed with ethanol and dried *in vacuo* to yield S-propyl-L-cysteine (630 mg, 47%); \( \delta_H \) 0.81 (3H, t, \( J = 7.4 \) Hz), 1.46 (2H, sext, \( J = 7.4 \) Hz), 2.43 (2H, t, \( J = 7.4 \) Hz), 2.86 (1H, dd, \( J = 7.6, 14.8 \) Hz), 2.96 (1H, dd, \( J = 4.3, 14.8 \) Hz), 3.76 (1H, dd, \( J = 4.3, 7.6 \) Hz); \( \delta_C \) 12.5 (1C, q), 22.2 (1C, t), 32.0 (1C, t), 33.4 (1C, t), 53.7 (1C, d), 173.1 (1C, s); HRESIMS: MH\(^+\), found 164.0740. \( C_6H_{14}NO_2S \) requires 164.0745.

**S-propyl-L-cysteine S-oxide (4)**

To a suspension of S-propyl-L-cysteine (300 mg, 1.84 mmol) in acetic acid (1.6 mL) was added a mixture of 35% aqueous hydroperoxide (0.17 mL) and acetic acid (1.7 mL) with stirring at room temperature. After 30 minutes, distilled water (10 mL) was added, and the mixture was lyophilized to yield S-propyl-L-cysteine S-oxide (4, 329 mg, quant.) as a colorless powder, which was obtained as a mixture of stereoisomers (major:minor = 55:45) that was used without further purification; \( \delta_H \) 0.93 (3H, t, \( J = 7.4 \) Hz), 1.65 (2H, m), 2.85 (2H, m), 3.08 (0.45H, dd, \( J = 7.8, 14.0 \) Hz), 3.25 (1.1H, d, \( J = 5.9 \) Hz), 3.32 (0.45H, dd, \( J = 6.0, 14.0 \) Hz), 4.09 (0.45H, dd, \( J = 6.0, 7.8 \) Hz), 4.21 (0.55H, t, \( J = 5.9 \) Hz); \( \delta_C \) 12.4 (1C, q), 15.8
(1C, t), 50.3 (0.55C, d), 50.5 (0.55C, t), 50.9 (0.45C, t), 51.1 (0.45C, d), 53.7 (1C, t), 171.4 (1C, s); HRESIMS: MH^+ found 180.0702. C_{6}H_{14}NO_{3}S requires 180.0694.

**Preparation of worm suspensions**

Suspensions of *D. destructor* were obtained from infected *A. sativum* bulbs using a modified Bearmann funnel technique\textsuperscript{16}. The bulbs, harvested from our experimental fields, were sterilized successively with aqueous 80% EtOH and aqueous 10% NaClO. The bulbs were hashed with a razor and soaked in sterile water on a funnel covered with a tissue for one night to obtain the worm suspension. The suspension was adjusted to 10^4 nematodes/mL with sterile water for the bioassay.

The suspension of *C. elegans* was maintained using standard procedures\textsuperscript{17}, and was prepared in the same manner as described above.

**Attracting activity bioassay protocol**

Ten milliliters of an agarose (0.6%) solution dissolved in boiling water was poured into each petri dish (50 mm\(\phi\)). After cooling, four holes (ca 0.2 cm\(^3\)) were scooped out crosswise with a cork bower (5 mm\(\phi\)). The tested compounds were dissolved in a 0.6% agarose solution, and the solution (200 \(\mu\)L) was loaded into a hole in the plate. Twenty \(\mu\)L of the worm suspension, containing 100 worms, was loaded on the center of the dish, which was then kept in the dark at 25°C overnight. Three dishes were prepared for each test sample. The worms on the agar were counted under the stereomicroscope. The nematode attractant activity was calculated as “Chemotaxis Index = \{(the number of worms in the tested area) - (the number of worms in the control area)\} / (the total number of worms)”.

**Statistical analysis**
All the results for bioassays are expressed as mean ± standard deviation (SD). Statistical analyses were done using R software (ver 3.6.1). Means comparisons were carried out using Dunnett’s test ($p < 0.05$ (*) or $p < 0.01$ (**)) after confirming normality by F-test. It was necessary to compare each attracting activity of several chemical-treated plates to the activity of untreated control plate in this paper. Thus, Dunnett’s test was adopted as the statistical analysis method.

**Results and discussion**

**Detection of attractant activity in *A. sativum* bulb extract**

An aqueous extract of *A. sativum* was prepared, and the nematode attractant activity of the extract was assessed with a bioassay, based on population chemotaxis using *D. destructor* eluted from infested *A. sativum* bulbs (Fig. 1a). However, the attractant activity of the extract was not detected at 1 g fresh weight equivalent (FW)/mL. Eventually, we found that *A. sativum* bulb juice prepared in 50 mM aqueous CaCl$_2$ displayed the attractant activity at 0.25 g FW/mL.
The flavor of *A. sativum* is well-known and is released when the raw material is crushed. The flavor is due to several organosulfur compounds generated through the enzymatic cleavages of S-alk(en)yl-L-cysteine S-oxides, such as alliin (2), by alliinase (cysteine sulfoxide lyase: E.C.4.4.1.4.)\(^{19,20}\). In the course of standard extraction procedures, alliinase cleaves S-alk(en)yl-L-cysteine S-oxides rapidly, leading to the absence of these compounds in the extract. It has been reported that sodium chloride is essential for preserving the enzymatic activity of alliinase, and calcium salts causes the enzyme to become unstable\(^{21}\). Thus, the sensitivity of S-alk(en)yl-L-cysteine S-oxides to alliinase suggested it to be the most plausible candidate for the attractant. This was used to ascertain whether the attractant in *A. sativum* could be decomposed by alliinase (Fig. 1b). Since alliinase is unstable in acid...

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**Fig. 1.** (a) Nematode (*D. destructor*) attractant activities of *A. sativum* extracts {H\(_2\)O (1 g FW/mL), aqueous CaCl\(_2\) (0.25 g FW/mL)} ; (b) Nematode (*D. destructor*) attractant activities of alliinase-inactive and active *A. sativum* bulb extracts. Each value represents the mean of the chemotaxis index ± SD (*N* = 3) Asterisks (*) and **(p < 0.05 and p < 0.01 versus control groups (Dunnett’s test), respectively.**}
conditions, an alliinase-inactive extract was prepared in 50% MeOH containing 1% TFA. The attractant activity of the alliinase-inactive extract was detected at 0.02 g dry weight equivalent (DW)/mL. In contrast, the alliinase-active extract did not display the activity even at a ten-fold greater concentration (0.2 g DW/mL). Hence, the attractant in *A. sativum* bulbs was suggested to be alliinase-sensitive S-alk(en)yl-L-cysteine S-oxide, such as compound 2.

**Preparations of alliin analogs tested in this study**

Fig. 2 shows the organosulfur compounds tested in this study. S-Propyl-L-cysteine S-oxide (4) was prepared in 47% yield from L-cysteine through S-propylation (propyl bromide, NaOH, EtOH) and successive oxidation (H_2O_2). Other compounds were obtained from the Aomori Prefectural Industrial Technology Research Center^{22,24}.

![Fig. 2. Structures of methiin (1) and its analogs.](image)

**Identification of methiin as the attractant in *A. sativum* bulb**

alliin (2, S-(2-propenyl)-L-cysteine S-oxide) is the most abundant sulfoxide found in *A. sativum*. Moreover, *A. sativum* contains methiin (1) and isoalliin (3, S-(E-1-propenyl)-L-
cysteine S-oxide). These sulfoxides undergo rapid enzymatic cleavage with the help of alliinase. The nematode attractant activities of compounds 1–3 were investigated first (Fig. 3). Compound 1 showed potent nematode attractant activity at 1 μM concentration. In contrast, neither compound 2 nor compound 3 showed the activity, even at a ten-fold greater concentration (10 μM). In a recent study, one of the authors developed a simultaneous quantification protocol for flavor precursors in *A. sativum*. Compound 1 was reported to account for 0.18–0.59% of dried *A. sativum* bulbs, corresponding to 4.2–13.9 mmol/kg FW.

For the present study, quantification experiments for compound 1 using LC/ESIMS demonstrated that the alliinase-inactive extract included 0.075% of compound 1, corresponding to 1.8 mmol/kg FW, whereas this was not detected in alliinase-active extract. Thus, *A. sativum* proved to significantly contribute to the level of compound 1 in the bulbs, which was sufficient 1 to attract *D. destructor* into *A. sativum* bulbs.
Attractant activity comparing study of methiin with its analogs

Although genomic analyses of D. destructor are being studied, the chemotactic mechanism of D. destructor still remains unclear. In this case, labeled analogs with attractant activity could provide critical clues for disclosing the molecular mechanism of chemotaxis. Allium plants include various organosulfur compounds besides 1–3. Thus, attractant activities of compound 1 analogs were examined to identify the essential structural motif for

Fig. 3. Nematode (D. destructor) attractant activities of methiin (1) and its analogs. The data indicates that the sulfoxide functionality is essential for attractant activity and that the S-alkyl moiety has a potential for structural modification. Each value represents the mean of the chemotaxis index ± SD (N = 3). Asterisks (**) show p < 0.01 versus control groups (Dunnett’s test).
their attracting activity. S-Propyl-L-cysteine S-oxide (4) exhibited the same potent activity (1 
µM) as compound 1. Compound 4 has been found in Chinese chives (Allium tuberosum L.)30 and Allium cepa31, but has not been identified in A. sativum23. In contrast, cycloalliin (8, 5-
methylthiomorpholine-3-carboxylic acid S-oxide) resistant to enzymatic cleavage32 did not 
show attractant activity. Therefore, in regard to acyclic saturated functionality, structural 
modifications on sulfur retained the activity. Allium plants have been known to possess 
considerable amounts of N-γ-glutamylated S-alk(en)yl-L-cysteines, which are often 
considered as storage forms of S-alk(en)yl-L-cysteine S-oxides because they are not sensitive 
to alliinase19. The activity of N-γ-glutamylated methiin (5) was investigated, and the structural 
modification resulted in a loss of attractant activity. This result corresponded with the 
sensitivity to alliinase described above. Furthermore, sulfide analog 9 did not show activity, 
indicating that the sulfoxide moiety essentially contributed to the activity. The loss of activity 
by this structural modification was confirmed in the bioassays of other analogs (6, 7, and 10– 
13). Therefore, our activity comparing study demonstrated that the sulfoxide functionality was 
necessary for attractant activity and that S-alkyl moiety in compound 1 has a potential for 
structural modification.

The attractant activity of methiin against C. elegans

The nematode Caenorhabditis elegans is a model organism for neuronal mechanism 

studies, including chemotactic behavior. Several attractants and repellants for C. elegans have 
been reported, thus enabling the identification of their chemosensory neurons33-35. The 
attractant activity of compound 1 to C. elegans was investigated, and detected at 10 µM, 
which is equivalent to the activity of diacetyl18, a well-known C. elegans attractant (Fig. 4). 
The neuronal system of D. destructor has not been established as well as that of C. elegans.
Further investigation of compound 1 as an attractant for *C. elegans* would facilitate the understanding of the neuronal system of *D. destructor*.

![Graph showing nematode attractant activity](image)

**Fig. 4.** Nematode (*C. elegans*) attractant activity of methiin (1). Each value represents the mean of the chemotaxis index ± SD (*N* = 3). Asterisk (**) shows *p* < 0.01 versus control groups (Dunnett’s test).

The study investigates the damage of *D. destructor* nematode to garlic, which greatly affects agriculture and is a serious threat to garlic production. This paper reveals that methiin, an abundant constituent in *A. sativum* bulbs, possesses sufficient potential to attract *D. destructor* into *A. sativum* bulbs. Furthermore, the activity comparing study of methiin with its analogs demonstrated that sulfoxide functionality was essential for attractant activity. Xu *et al.* reported an attractant effect of sweet potato root exudates. It is possible that *D. destructor* similarly approaches *A. sativum* plants under the guidance of methiin secreted from *A. sativum* roots. However, these results were insufficient to conclude that methiin attracts the worms in garlic fields, due to the lack of evidence for the secretion of methiin from *A. sativum*. In order to disclose the chemical communication between the pathogenic nematode *D. destructor* and the host plant *A. sativum*, chemical analysis of root exudate from *A. sativum* is underway. In addition, this study suggests that methiin is one of the major constituents in well-established food (*A. sativum*) and could be used as a nematode attractant without causing damage to surrounding organisms. It is plausible for a nematode trap using the attractant to
become a new type of pesticide in *A. sativum* production. Such experiments are ongoing in our experimental fields.

**Conclusions**

Damage to *A. sativum* caused by nematodes *D. destructor* is leading to a great loss in garlic production. After invasion of *D. destructor* into garlic bulbs, the nematodes drastically increase population along with the rotting of bulbs. The characteristic phenomenon led to be conceived that nematode attractants are present in the bulbs. Based on this hypothesis, chemical investigations were performed to explore a nematode attractant in *A. sativum* bulbs, which resulted in the identification of methiin as an attractant. Bioassay and quantification experiments of methiin in extracts of *A. sativum* bulb led to the conclusion that methiin possesses sufficient potential to attract *D. destructor* into *A. sativum* bulbs. Moreover, methiin was revealed to attract *Caenorhabditis elegans*.

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**References**

**Figure Captions**

**Fig. 1.** (a) Nematode (*D. destructor*) attractant activities of *A. sativum* extracts {H$_2$O (1 g FW/mL), aqueous CaCl$_2$ (0.25 g FW/mL)}; (b) Nematode (*D. destructor*) attractant activities of alliinase-inactive and active *A. sativum* bulb extracts. Each value represents the mean of the chemotaxis index ± SD (*N* = 3). Asterisks (* and **) show *p* < 0.05 and *p* < 0.01 versus control groups (Dunnett’s test), respectively.

**Fig. 2.** Structures of methiin (1) and its analogs.

**Fig. 3.** Nematode (*D. destructor*) attractant activities of methiin (1) and its analogs. The data indicates that the sulfoxide functionality is essential for attractant activity and that the S-alkyl moiety has a potential for structural modification. Each value represents the mean of the chemotaxis index ± SD (*N* = 3). Asterisks (**) show *p* < 0.01 versus control groups (Dunnett’s test).

**Fig. 4.** Nematode (*C. elegans*) attractant activity of methiin (1). Each value represents the mean of the chemotaxis index ± SD (*N* = 3). Asterisk (**) shows *p* < 0.01 versus control groups (Dunnett’s test).