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Potent Nematicidal Activity of Phthalaldehyde, Salicylaldehyde, and Cinnamic Aldehyde against *Meloidogyne incognita*

Pierluigi Caboni,*[,†] Nadhem Aissani,[†] Tiziana Cabras,[†] Andrea Falqui,[#] Roberto Marotta,[#] Barbara Liori,[†] Nikoletta Ntalli,[†] Giorgia Sarais,[†] Nicola Sasanelli,[§] and Graziella Tocco[†]

[†]Department of Life and Environmental Sciences, University of Cagliari, Via Ospedale 72, 09124 Cagliari, Italy

[§]Institute for Plant Protection, National Research Council, Via G. Amendola 122/D, 70126 Bari, Italy

[#]Nanochemistry, Istituto Italiano di Tecnologia, Via Morego 30, 16163 Genova, Italy

ABSTRACT: The nematicidal activity of selected aromatic aldehydes was tested against the root knot nematode *Meloidogyne incognita*. The most active aldehyde was phthalaldehyde (**1**) with an EC_{50} value of 11 ± 6 mg/L followed by salicylaldehyde (**2**) and cinnamic aldehyde (**3**) with EC_{50} values of 11 ± 1 and 12 ± 5 mg/L, respectively. On the other hand, structurally related aldehydes such as 2-methoxybenzaldehyde (**21**), 3,4-dimethoxybenzaldehyde, and vanillin (**23**) were not active at the concentration of 1000 mg/L. By liquid chromatography−mass spectrometry the reactivity of tested aldehydes against a synthetic peptide resembling the nematode cuticle was characterized. At the test concentration of 1 mM, the main adduct formation was observed for 3,4-dihydroxybenzaldehyde (**22**), 2-methoxybenzaldehyde (**21**), and 3,4-dimethoxybenzaldehyde. Considering that 2-methoxybenzaldehyde (**21**) and 3,4-dimethoxybenzaldehyde were not active against *M. incognita* in in vitro experiments led us to hypothesize a different mechanism of action rather than an effect on the external cuticle modification of nematodes. When the toxicity of the V-ATPase inhibitor pyocyanin (**10**) was tested against *M. incognita* J2 nematodes, an EC_{50} at 24 h of 72 ± 25 mg/L was found. The redox-active compounds such as phthalaldehyde (**1**) and salicylaldehyde (**2**) may share a common mode of action inhibiting nematode V-ATPase enzyme. The results of this investigation reveal that aromatic redox-active aldehydes can be considered as potent nematicides, and further investigation is needed to completely clarify their mode of action.

KEYWORDS: *aromatic aldehydes, botanical pesticide, nematicide, reactive oxygen species, advanced glycation end products, V-ATPase, redox-active aldehydes*

INTRODUCTION

Synthetic nematicides have long had application in the protection of agricultural crops and in the control and management of root-knot nematodes (RKN), *Meloidogyne* spp. being the most common and widespread group of nematodes in the world.[1,2] Root-knot nematodes are soilborne root parasites of different crop species that importantly affect the host plant physiology and cause annually U.S. $100 billion of crop losses in fruit and vegetable production.[3] Plant growth impairment caused by *Meloidogyne* spp. to vegetable crops is influenced by nematode species and physiological race as well as the initial nematode population density in the soil at sowing or transplanting.[4] Recent European legislation has deeply revised and restricted the use of pesticides on agricultural crops, focusing attention on animal and human health and environmental safety.[5] Plant protection from plant parasitic nematodes and soilborne plant pathogens should therefore rely on alternative control strategies that are both economically sustainable and environmentally sound at the same time.

At present, in Europe, there are registered nematicides available for the control of RKN, that is, fosthiazate, fenamiphos, oxamyl (no fumigants; they can be applied at transplant or sowing) and dazomet, 1,3-dichloropropene, and metham sodium or potassium (fumigants; they must be applied to the soil at least 4 weeks before sowing or transplant because of their phytotoxicity).[6] On the other hand, methyl bromide, a traditional fumigant, was phased out because of its role in ozone depletion.[7] The control of nematodes on different vegetables has been lately performed by botanical extracts, such as sesame stalk or oil, neem cake, crab shell meal, quillaja, and tannins.[8−12] In particular, plant-derived nematicidal compounds have lately gained importance, specifically aldehydes such as furfural (**24**) from *Melia azedarach*, (*E*,*E*)-2,4-decadienal and (*E*)-2-decenal from *Ailanthus altissima* and ketones such as 2-undecanone from *Ruta chalepensis*, were greatly effective in controlling RKN in vitro and in pot experiments.[13−16] Furthermore, virtually nothing is known regarding the mode of action of nematicidal aldehydes, ketones, and acids. This research examined redox-active aldehyde nematicides using the model nematode *Meloidogyne incognita*. The latter is highly amenable to large-scale screening operations due to the easy rearing system on tomato plants.

The nematode cuticle, well reviewed in the *Caenorhabditis elegans* model,[17] is a multifunctional exoskeleton. It is a highly impervious barrier between the animal and its environment, and it is essential for maintenance of body morphology and integrity and for osmoregulation, and it has a critical role in locomotion via attachments to body-wall muscles.[18−21] The cuticle collagens are encoded by a gene family with over 170

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members.[18] They have a characteristic tripeptide structure of short interrupted blocks of glycine−proline−hydroxyproline sequence flanked by conserved cysteine residues and can be grouped into families according to homology.[22] Taking into account the feasible reaction of small molecules having electrophilic properties with nucleophilic amino acid residues of the proteins, that is, cysteine, we investigated the chemical reaction between some selected compounds and the nematode's cuticle peptides to better understand their potential toxicity.

Reactivity of electrophilic aldehydes toward cuticle or skin peptides has been investigated qualitatively for individual chemicals by directly detecting and characterizing adducts between the peptides and the chemical using liquid chromatography−mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR).[23−25] With an LC-MS platform, constituents of a reaction mixture can be separated, and full scan mass spectra for ions with mass/charge ratio (m/z) are acquired during the chromatographic run. Consequently, an LC-MS analysis of the products after incubation with peptide and test compounds allows selective monitoring of the test compound and testing of different molecular weight adducts. These approaches are widely used to predict skin sensitization of test compounds toward skin peptides and proteins.[26−29] In parallel, it was assayed by cysteine reduction/alkylation experiments, the involvement of the cysteine residues in the adduct formation between the tested compounds and the peptide.

It is well-known that glycation of biological proteins can lead to the formation of advanced glycation end products (AGEs) that have a propensity to generate toxic reactive oxygen species (ROS).[30,31] In the same fashion, gossypol, a triterpenoid aldehyde, isolated from cotton plants was shown to inhibit mitochondrial electron transfer and stimulate generation of ROS.[32] Thus, for these reasons it was hypothesized that ROS generation produced by redox-active aldehydes may have a crucial role in the inhibition of vacuolar-type proton translocating ATPase (V-ATPase). The latter target is an enzyme complex that pumps protons across membranes, energized by ATP hydrolysis. V-ATPases, depending on their subcellular localization and orientation in the membrane, can acidify intracellular compartments[33] or the extracellular environment.[34] Consequently they are involved in nematode nutrition osmoregulation, cuticle synthesis, neurobiology, and reproduction.[35] Osmoregulation can be critical for nematodes as maintenance of homeostasis and purging of toxins would be essential normal and hostile environments.[36] On the basis of in vitro studies, V-ATPases from mammals, fungi, and plants are subject to oxidative inactivation, whereas activity can be restored by using a reducing agent.[37−39] Moreover, oxidative inhibition of the V-ATPase is ascribed to disulfide bond formation between conserved cysteine residues at the catalytic site.[40] Furthermore, V-ATPase is inhibited by the antibiotics bafilomycin A1 and concanamycin A and inactivated by the phenazine pyocyanin (**10**).[41,42]

In summary, this paper reports information on the potential efficacy of low molecular weight nematicides obtained using a *Meloidogyne* testing system. The objectives of this research were (1) to define the optimal condition for testing low molecular weight nematicides; (2) to evaluate 32 candidate compounds using the bioassay and rank them on the basis of relative efficacy; (3) to assess structure−activity relationships among the nematicides themselves that affect relative toxicity; (4) to evaluate the cuticle surface damages after treatment with selected active compounds; and (5) to evaluate the molecular mechanism of the reactivity of selected chemicals with a synthetic nematode cuticle peptide.

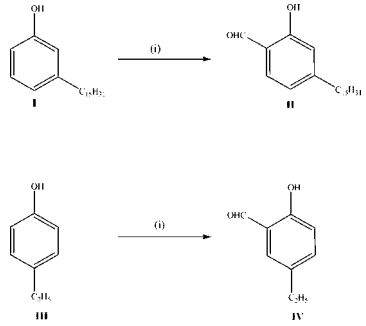
MATERIALS AND METHODS

Chemicals. All of the chemicals were of purity >98%. Phthalaldehyde (**1**), salicylaldehyde (**2**), cinnamic aldehyde (**3**), 2-naphthaldehyde (**4**), piperonal (**7**), 3-hydroxybenzaldehyde (**6**), citral (**9**), pyocyanin (**10**), 4-hydroxybenzaldehyde (**11**), 2-nitrobenzaldehyde (**12**), phenylacetaldehyde (**13**), benzaldehyde (**14**), cinnamic acid (**15**), helicin (**16**), 3,4-methylenedioxy acetophenone (**17**), catechol (**18**), benzoic acid-2-hydroxy methyl ester (**19**), 3-formylbenzoic acid (**20**), 2-methoxybenzaldehyde (**21**), 3,4-dihydroxybenzaldehyde (**22**), 3,4-dimethoxybenzaldehyde, vanillin (**23**), furfural (**24**), abamectin, levamisole, ouabain, (*E*,*E*)-2,4-decadienal, and fosthiazate as well as Tween 20 and dimethyl sulfoxide were obtained from Sigma-Aldrich (Milano, Italy). Isophthalic dicarboxaldehyde (**5**) was obtained from Alfa Aesar. Methanol and water were of HPLC grade.

The peptide *N*-acetyl-EEQCTSCVQLQCP (88% of purity) was purchased from Genscript USA Inc.

General Procedure for the Formylation of Phenols I and III.[43] A solution of the appropriate phenol (4.09 mmol) in 2.5 mL of 10 N NaOH was heated to 65 °C. After that, 1 mL of $CHCl_3$ was slowly added to the mixture, which was then heated at reflux in chloroform for 2 h. After cooling, the mixture was acidified to pH 1 with concentrated HCl, the organic layer collected, and the aqueous phase extracted with $CHCl_3$. The combined organic phase, previously dried on Na_2SO_4, was concentrated to give a crude product, which was purified by flash chromatography on silica gel (Scheme 1).

Scheme 1. Reagents and Conditions[a]



[a](i) NaOH 10 N, $CHCl_3$, 65 °C, 2 h.

2-Hydroxy-4-pentadecylbenzaldehyde: ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.95 (s, 1H), 7.62 (s, 1H), 6.89 (d, J_3 = 9 Hz, 1H), 6.56 (d, J_3 = 9 Hz, 1H), 1.55 (m, 28H), 1.16 (t, 3H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 191.2, 162.3, 145.1, 134.8, 121.2, 36.7, 30.2, 16.9.

*5-Ethyl-2-hydroxybenzaldehyde (**8**):* ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.0 (s, 1H), 7.52 (s, 1H), 7.21 (d, J_3 = 9 Hz, 1H), 6.97 (d, J_3 = 9 Hz, 1H), 2.65 (q, J_3 = 11 Hz, 2H), 1.32 (t, J_3 = 11 Hz, 3H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 189.1, 172.0, 163.5, 141.0, 122.4, 112.3, 33.5, 17.7.

General Procedure for Aldehyde Immobilization on PEG. All PEG samples (Aldrich) were melted under vacuum at 90 °C for about 45 min before use to remove any trace of moisture. After reaction, the crude mixture was concentrated under vacuum to eliminate the solvent, and then 6−7 mL of CH_2Cl_2 was added to completely dissolve the residue. The obtained mixture was added to Et_2O (50 mL/g polymer) cooled at 0 °C. The obtained suspension was filtered through a sintered glass filter, and the solid obtained was repeatedly washed on the filter with pure Et_2O. All of the samples have been crystallized from isopropyl alcohol, to eventually eliminate the excess of the polar reagents or the byproducts. The yields of PEG-supported

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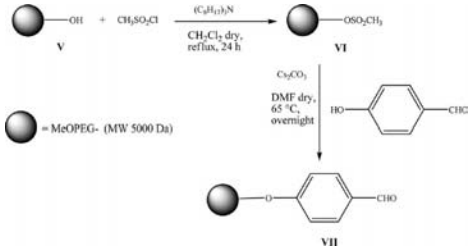
compounds were determined by weight. The indicated yields were for pure products after crystallization from isopropyl alcohol. Their purity was confirmed by 500 MHz ^{1}H NMR analysis in $CDCl_3$ with a presaturation of the methylene signals of the polymeric support at 3.60 ppm. When the NMR spectra were recorded, a relaxation delay of 6 s and an acquisition time of 4 s were used to ensure complete relaxation and accuracy of integration. The integrals of the signals of the PEG CH_2OCH_3 fragment at δ 3.30 and 3.36 were used as internal standards.

4-OPEG-benzaldehyde. The synthetic procedure for the preparation of PEG polymer-bound aldehyde started by mesylating the monomethyl ether of poly(ethylene glycol) (**V**) with M_w = 5000 Da. Then, the 4-hydroxybenzaldehyde (**11**) was anchored to PEG-mesylate 2 (**VI**), giving final product **VII** (Scheme 2).[44]

Scheme 2. Reagents and Conditions[a]



[a](i) mesyl chloride, trioctylamine, dichloromethane dry, reflux, 24 h. (ii) Cs_2CO_3 dimethylformamide dry, 4-hydroxybenzaldehyde, 65 °C, overnight.

*PEG monomesylate (**V**):* ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.08 (s, 3H).

*4-OPEG-benzaldehyde (**VII**):* ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.87 (s, 1H), 7.82 (d, J_3 = 7 Hz, 2H), 7.015 (d, J_3 = 7 Hz, 2H).

Effect of Different Aromatic Aldehydes on J2 Motility. The solutions of the aromatic aldehydes were tested on J2 motility of *M. incognita* at different concentration ranges for EC_{100s} at 1 h and EC_{50s} at 1 day of calculation. The compounds used for the paralysis experiment were phthalaldehyde (**1**), salicylaldehyde (**2**), cinnamic aldehyde, 2-naphthaldehyde (**4**), piperonal (**7**), 3-hydroxybenzaldehyde (**6**), citral (**9**), pyocyanin (**10**), 4-hydroxybenzaldehyde (**11**), 2-nitrobenzaldehyde (**12**), phenylacetaldehyde (**13**), benzaldehyde (**14**), cinnamic acid (**15**), helicin (**16**), 3,4-methylendioxyacetophenone (**17**), catechol (**18**), benzoic acid-2-hydroxy methyl ester (**19**), 3-formylbenzoic acid (**20**), 2-methoxybenzaldehyde (**21**), 3,4-dihydroxybenzaldehyde (**22**), vanillin (**23**), furfural (**24**), abamectin, levamisole, ouabain, (*E*,*E*)-2,4-decadienal, fosthiazate, isophthalic dicarboxaldehyde (**5**), 5-ethyl-2-hydroxybenzaldehyde (**8**), 2-hydroxy-4-pentadecylbenzaldehyde, 3,4-dimethoxybenzaldehyde, and 4-OPEG-benzaldehyde.

Stock solutions were prepared in methanol to overcome insolubility, whereas Tween 90 in distilled water was used for further dilutions. Final concentrations of methanol in each well never exceeded 1% v/v, as previously reported.[15] Distilled water as well as a mixture of water and Tween (0.2% v/v) (carrier control) served as controls. In all cases, working solutions were prepared containing double the test concentration and mixed in CellstarR 96-well cell culture plates (Greiner bio-one) at a ratio of 1:1 v/v with suspensions of 25 J2 added to each well. Multiwell plates were covered to prevent evaporation and maintained in the dark at 28 °C.

Juveniles were obtained by an Italian population of *M. incognita* (Kofoid et White) Chitw. reared for 2 months on tomato (*Solanum lycopersicum* L.) cv. Rutgers in a glasshouse at 25 ± 2 °C. *Meloidogyne* species was identified by preparing perineal patterns from females (Figure 1). Egg masses from infested tomato roots were collected and their eggs hatched by distilled water (natural hatching agent) to obtain second-stage juveniles (J2) to use in the experiment in groups of 25 J2 (Figure 2). Juveniles were observed with the aid of an inverted microscope (Zeiss, 3951, Germany) at 10× after 1 and 24 h and were

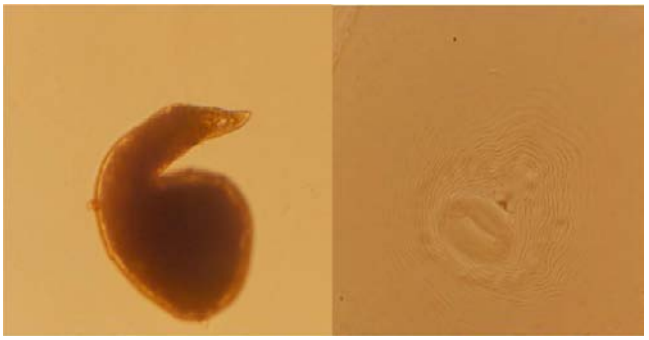


Figure 1. Female of *Meloidogyne incognita* (left) and perineal pattern (right).

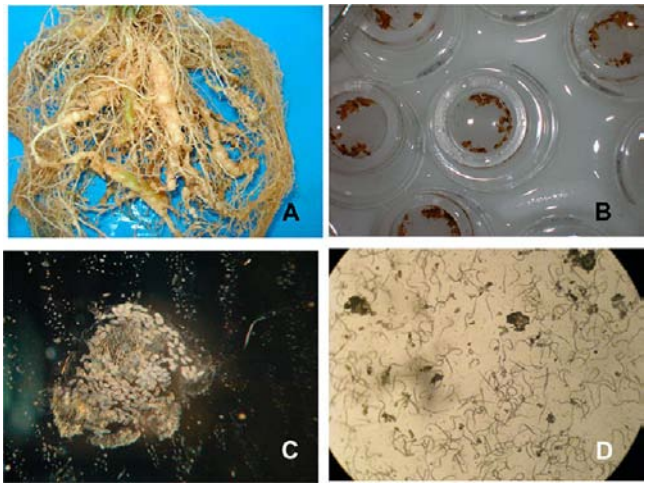


Figure 2. *Meloidogyne incognita* infested tomato root (A) with batches of egg masses for egg hatch (B), eggs inside egg mass (C), and hatched juveniles (D).

successively ranked into two distinct categories: motile or paralyzed. Subsequently, nematodes were moved to plain water after washing in tap water through a 20 μm pore screen to remove the excess of extracts. Juveniles that did not display motility after transfer to distilled water for 1 h and when stimulated with a needle on the body were considered to be paralyzed. Numbers of motile and paralyzed J2 were counted.

Statistical Analysis. Treatments of paralysis experiments were replicated five times, and each experiment was performed twice. The percentages of paralyzed J2 in the microwell assays were corrected by elimination of the natural death/paralysis in the water control according to the formula corrected % = [(mortality % in treatment − mortality % in control)/(100 − mortality % in control)] × 100, and they were analyzed (ANOVA) combined over time. Because ANOVA indicated no significant treatment by time interaction, means were averaged over experiments. Corrected percentages of paralyzed J2 treated with test compounds were subjected to nonlinear regression analysis using the log−logistic equation proposed by Seefeldt et al.:[45] $Y = C + (D - C)/\{1 + \exp[b\ (\log(x) - \log(EC_{50})]\}$, where C = the lower limit, D = the upper limit, b = the slope at the EC_{50}, and EC_{50} = the test compound concentration required for 50% death/paralysis of nematodes after elimination of the control (natural death/paralysis). In the regression equation, the test compound concentration (% w/v) was the independent variable (x) and the paralyzed J2 (percentage increase over water control) was the dependent variable (y). The mean value of the five replicates per test concentration and immersion period was used to calculate the EC_{50} value.

SEM Characterization. Freshly hatched J2 nematodes were treated for 24 h by immersion in two 100 μL solutions containing respectively 100 mg/L of salicylaldehyde (**2**) and 100 mg/L of (*E*,*E*)-

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2,4-decadienal. Thereafter, both treated and untreated nematodes, the latter used as negative controls, were postfixed in a solution of osmium tetroxide (1%) in 0.1 M cacodylate buffer for 1 h and extensively washed in distilled water. The specimens were then rapidly frozen in slush nitrogen (SN2). After immersion in SN2, the specimens were rapidly transferred to the cryochamber cold stage of a K750X Freeze Drier (Emitech LtD., UK) and freeze-dried under vacuum overnight, allowing the temperature to gradually rise from −70 °C to room temperature. The specimens were then coated with 10 nm gold by a sputter coater (108auto/SE Cressington, UK) and imaged using the secondary electron signal by a Jeol JSM 7500FA scanning electron microscope (Jeol Ltd., Japan), equipped with a cold field emission gun and working at an acceleration voltage of 15 kV.

Peptide Reactivity with Different Aldehydes. 3,4-Dihydroxybenzaldehyde (**22**), 2-methoxybenzaldehyde (**21**), 3,4-dimethoxybenzaldehyde, salicylaldehyde (**2**), piperonal (**7**), benzaldehyde (**14**), and isophthalic dicarboxaldehyde (**5**) were used to test the reactivity of the synthetic peptide *N*-acetyl-EEQCTSCVQLQCP. Stock solutions of 40 mM aldehydes were prepared in 200 mM Tris-HCl buffer, pH 7.4, and that of the peptide (1 mM) in water. Fifty microliters of the reaction mixtures contained 1 mM, 5 mM, and in some cases 15 mM of the aldehyde and 0.5 mM of the peptide in 100 mM Tris-HCl buffer, pH 7.4. The reactions were performed in duplicate at 25 °C in the dark for 24 h and stopped by the addition of aqueous TFA (0.1%, final concentration). The control sample contained only the peptide at the same concentration. Ten microliters of the reaction mixtures were added with 90 μL of 0.1% TFA and immediately analyzed by HPLC-ESI-MS or stored at −20 °C until the analysis.

LC-MS Detection of Reaction Products. HPLC-ESI-MS analysis was performed using a Surveyor HPLC system (ThermoFisher, San Jose, CA, USA) connected by a T splitter to a diode array detector and to an LCQ Advantage mass spectrometer (ThermoFisher). The chromatographic column was a BioBasic-18 column (Thermo Scientific, USA), with 5 μm particle diameter (column dimensions 150 × 2.1 mm). The following solutions were utilized for the chromatographic separation: (eluent A) 0.056% aqueous TFA and (eluent B) 0.050% TFA in acetonitrile/water 80:20 (v/v). The gradient applied was linear from 0 to 40% of B in 30 min and from 40 to 100% of B in 10 min, at a flow rate of 0.30 mL/min. The T splitter addressed a flow rate of 0.20 mL/min toward the diode array detector and 0.10 mL/min toward the ESI source. During the first 5 min of separation the eluate was diverted to waste to avoid instrument damage due to the high salt concentration. The diode array detector was set at 214 and 276 nm. Mass spectra were collected every 3 ms in the positive ion mode. MS spray voltage was 5.0 kV and capillary temperature, 260 °C. Deconvolution of averaged ESI-MS spectra was automatically performed with MagTran 1.0 software.[46] Experimental mass values were compared with average theoretical values using the PeptideMass program available at http://us.expasy.org/tools. The relative abundances of the unmodified peptide and of the derivatives detected were determined by measuring the XIC (extracted ion current) peak area, when the signal/noise ratio was at least 5. The XIC analysis reveals the peak associated with the peptide of interest by searching along the total ion current chromatographic profile, the specific multiply charged ions generated at the source by the peptide. The area of the ion current peak is proportional to concentration, and under constant analytical conditions can be used for a label-free quantification.[47] For each tested aldehyde compound we calculated the relative percentage of the XIC peak area of every derivative with respect to the sum of the XIC peak area of all the derivatives.

Cysteine Reduction and Alkylation Experiments. Twenty microliters of the reaction mixtures (with salicylaldehyde (**2**) and 3,4-dihydroxybenzaldehyde (**22**)) and of the control sample containing 10 nmol of the probe peptide was submitted to cysteine reduction at 30 °C for 1 h in 50 mM Tris-HCl buffer, pH 8.6, containing 10 mM dithiothreitol (DTT) in a final volume of 40 μL. The reduced sample was alkylated in the dark for 45 min using 55 mM iodoacetamide (IAM), determining a Δmass value of +57.05 Da for each cysteine alkylated and subsequently analyzed by HPLC-ESI-MS.

RESULTS AND DISCUSSION

Several bioassay configurations were compared for exposing nematodes to test compounds. The optimal bioassay configuration permits 100% nematode survival for 48 h under control condition and involves placing nematodes in 400 μL 96-well plates and covering the plates with parafilm and keeping them in the dark at ambient temperature. Using the optimized bioassay conditions, five solvent carriers were tested for the relative toxicity to test nematodes: methanol, dimethyl sulfoxide, dichloromethane, Tween 20, and hexane. Only hexane and dichloromethane elicited 100% mortality, which was severe. No mortality was observed for the remaining solvents or untreated controls. Methanol was chosen as standard solvent for use in the nematicide bioassay.

Twenty-six low molecular weight compounds (Figure 3) with suspected nematicidal activity were identified and purchased from commercial sources or synthetically prepared and compared for toxicity with commercial nematicide. Test compounds were either dissolved or diluted in methanol at standard concentrations between 2000 and 10000 mg/L and applied to bioassay at the desired concentration after dilution with water or a mixture of water and Tween. These concentrations provided a linear concentration−mortality relationship for all nematicides tested.

Overall, the best performing nematicidal compounds were phthalaldehyde (**1**) and salicylaldehyde (**2**) (EC_{50} = 11 ± 6 and 11 ± 1 mg/L, respectively). These compounds were followed by cinnamic aldehyde and 2-naphthaldehyde (EC_{50} = 12 ± 5 and 13 ± 8 mg/L, respectively) and then isophthalic dicarboxyaldehyde (**5**) (EC_{50} = 15 ± 4 mg/L). Finally, the positive control fosthiazate, abamectin, and levamisole showed considerably higher toxicities than any experimental compounds (EC_{50} = 0.4 ± 0.3, 1.8 ± 0.9, and 4 ± 2 mg/L, respectively) (Table 1).

The data presented here show that structural factors contribute to the widely varying toxicities of low molecular weight nematicides. With respect to the aromatic compounds, two structure−activity relationship trends are apparent. First, when no other substituents are present in the benzene ring, the 1,2-dicarboxaldehyde was slightly more toxic if compared with isophthalic dicarboxyaldehyde. Second, the presence of a hydroxyl group in the ortho position to the formyl group conferred the highest activity if compared with the para position (4-hydroxybenzaldehyde (**11**)). In addition, when the hydroxyl group was substituted with a nitro group, the tested compound was 11 times less toxic than salicylaldehyde (**2**). Furthermore, when the hydroxyl group in the ortho position was glycated, as in helicin (**16**), the test compound lost completely its activity. The same behavior was observed for the methoxylation of 2-methoxybenzoaldehyde (**21**) and vanillin (**23**). Surprisingly, benzaldehyde (**14**) was not toxic at the tested concentration (250 mg/L), and these data are not in agreement with the previously reported findings of Ntalli,[48] probably because benzaldehyde readily undergoes oxidation by air. Moreover, the cinnamic acid (**15**) was not active if compared with the cinnamic aldehyde (**3**). Also, the 3,4-methylenedioxyacetophenone was not active if compared with 1,3-benzodioxole-5-carbaldehyde or piperonal (**7**) (EC_{50} = 44 ± 13 mg/L). To the best of our knowledge, no information concerning the mechanism of action and the selectivity against nematodes of our aromatic aldehydes is present in the

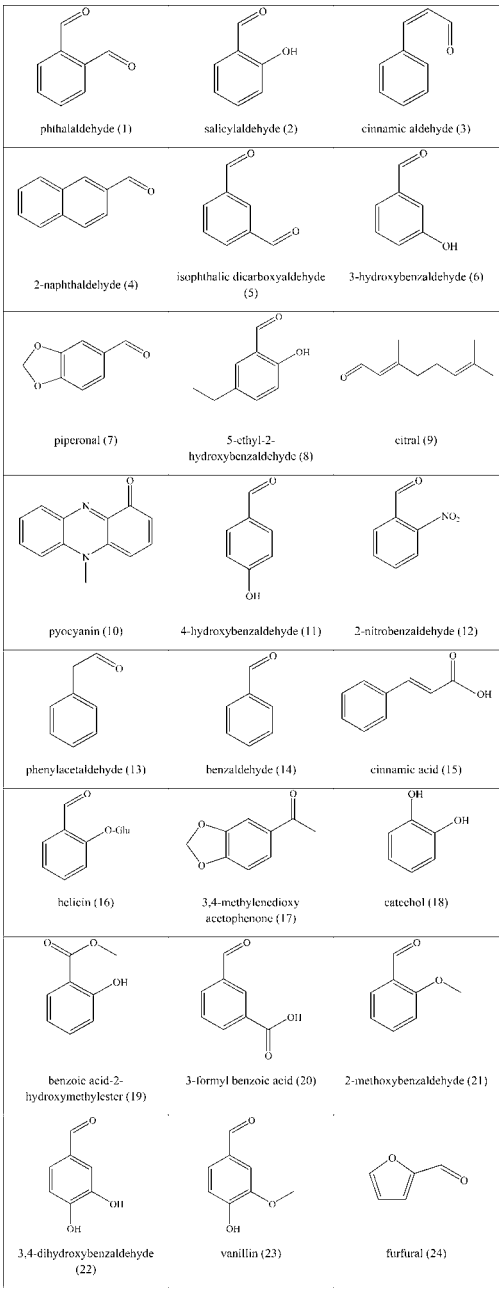

Figure 3. Chemical structures of selected nematicidal compounds.

literature. One factor that most aromatic aldehydes shared is acute nematode poisoning.

We previously reported that both treatments with furfural (**24**) and (*E*,*E*)-2,4-decadienal at 100 mg/L induced marked damages on the nematode well evidenced by environmental scanning electron microscopy (ESEM).[15] In this work we deepened our scanning electron microscopy (SEM) investigation of the nematode's cuticle after treatment with salicylaldehyde (**2**) and (*E*,*E*) 2,4-decadienal at 100 mg/L (Figure 4). Compared to the controls, the treated nematodes showed evident damages on the external cuticle, with marked macroscopic fractures (Figure 4, compare panels A with C and F). The cuticles of the control nematodes showed a typical annular wrinkled pattern (Figure 4A,B), which appeared to be smooth and stretched in the nematodes treated with salicylaldehyde (**2**) (Figure 4D,E) and (*E*,*E*) 2,4-decadienal (Figure 4G,H), probably as a consequence of their swollen and tumescent bodies. This diversity in the cuticle annular pattern might be caused by differences in the nematode body water content before fixation, although it is difficult to discern if the body swelling depended on a specific change in the osmolar efficiency occurring in the living nematodes and was caused by the tested compounds or by an unspecific post-mortem artifact. Intriguingly, besides these macroscopic differences, the fine morphology of the cuticle of the nematodes treated with salicylaldehyde (**2**) and (*E*,*E*) 2,4-decadienal did not differ significantly from that of the control ones, not presenting microfractures (Figure 4B,E,H), and then only nanometric fracture of the nematode cuticle could be masked by the conductive gold coating.

Furthermore, considering that 4-hydroxybenzaldehyde (**11**) was active against juveniles of *M. incognita* but the nature of this toxicity is mostly undefined, we prepared a structurally related compound with the same aromatic scaffold bearing a bulky PEGylated group in the para position. In in vitro tests, this compound did not show any activity, thus suggesting it was not

Table 1. Values of EC_{100} at 1 h and EC_{50} at 1 Day (n = 4) of Pure Compounds against *M. incognita* after Treatment

compound	EC_{100}, 1 h (mg/L ± SD)	EC_{50}, 1 day (mg/L ± SD)
phthalaldehyde (**1**)	>50	11 ± 6
salicylaldehyde (**2**)	>25	11 ± 1
cinnamic aldehyde (**3**)	>250	12 ± 5
2-naphthaldehyde (**4**)	>75	13 ± 7
isophthalic dicarboxaldehyde (**5**)	>100	15 ± 5
3-hydroxybenzaldehyde (**6**)	>250	31 ± 22
piperonal (**7**)	>100	44 ± 13
5-ethyl-2-hydroxybenzaldehyde (**8**)	>100	53 ± 22
citral (**9**)	>250	72 ± 27
4-hydroxybenzaldehyde (**11**)	500	75 ± 23
2-nitrobenzaldehyde (**12**)	>200	120 ± 25
phenylacetaldehyde (**13**)	1000	167 ± 45
benzaldehyde (**14**)	>100	>250
cinnamic acid (**15**)	>250	>250
helicin (**16**)	>250	>250
3,4-methylendioxy acetophenone (**17**)	>500	>500
catechol (**18**)	>500	>500
2-hydroxy-4-pentadecylbenzaldehyde (**II**)	>1000	>1000
benzoic acid-2-hydroxy methyl ester (**19**)	>1000	>1000
PEG-*O*-4-hydroxybenzaldehyde (**VII**)	>1000	>1000
3-formylbenzoic acid (**20**)	>1000	>1000
2-methoxybenzaldehyde (**21**)	>1000	>1000
3,4-dihydroxybenzaldehyde (**22**)	>1000	>1000
3,4-dimethoxybenzaldehyde	>1000	>1000
vanillin (**23**)	>1000	>1000
ouabain	>1000	>1000
pyocyanin (**10**)	>250	72 ± 25
fosthiazate	3 ± 1	0.4 ± 0.3
abamectin	>75	2 ± 1
levamisole	>20	4 ± 2
(*E*,*E*)-2,4-decadienal	>20	12 ± 2
furfural[a] (**24**)	11 ± 1	8 ± 1
oligomycin	>500	>500

[a]This compound showed a strong fumigant activity.

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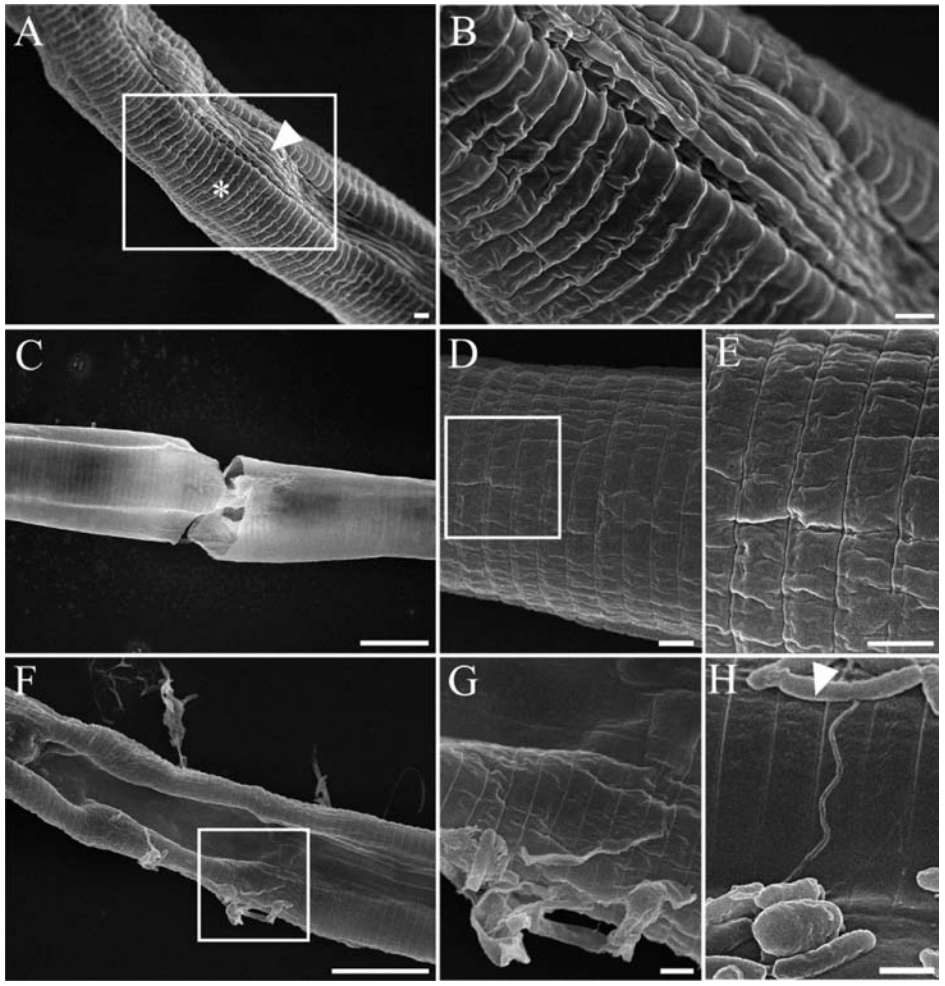

Figure 4. Scanning electron microscopy (SEM) micrographs of the cuticle of *Meloidogyne incognita* juveniles. (A, B) Untreated (control) specimens: (A) low magnification of a portion of *M. incognita* juvenile body surface (note the annulated cuticle cortical layer (asterisk) separated by several epicuticular longitudinal ridges (arrowhead)); (B) detail of the cuticle correspondent to the region boxed in panel A. (C–E) Body surface after treatment with salicylaldehyde (**2**): (C) low magnification showing a macroscopic fracture through the nematode body wall; (D) detail of the cuticle annular pattern; (E) higher magnification of several adjacent annuli in the cuticle region boxed in panel D. (F–H) Body surface after treatment with (*E*,*E*)-2,4-decadienal; (F) low magnification of the nematode body surface showing conspicuous damages at the cuticular layers; (G) detail of the damaged cuticle annular region boxed in panel F; (H) higher magnification of the cuticle annular region (note several rod-shaped bacteria on adjacent cuticular annuli (arrowhead)). Scale bars are 1 μm for panels A, B, D, E, G, and H and 10 μm for panels C and F.

able to diffuse through the nematode cuticle, most likely signifying an internal biochemical target of tested aldehydes.

Taking into account the nematode toxicity experiments with different aldehydes and the macroscopic cuticle fractures observed by SEM, we hypothesize that the potential mechanism of action of the tested aromatic aldehydes might be related to the alteration of the external cuticle or an internal biochemical target might be involved.

First, to understand the toxicity mechanism, we decided to prove the formation of a covalent adduct between aromatic aldehydes and endogenous proteins and/or peptides mimicking the nematode cuticle.

The sequence of the probe peptide utilized in the assay corresponded to the 100–112 residues of collagen 3 (Swiss-Prot code O01945) present in the cuticle of *M. javanica* and coded by the Mjcol-3 gene.[49] The peptide sequence was chosen according to the following criteria: (1) it covered a region of Mj collagen 3 containing from three to five cysteine residues involved in the disulfide bridge formation during the collagen assembly;[49,50] (2) cysteine- or lysine-containing peptides are usually used for mechanistic understanding of the reactivity of chemicals toward collagen peptides in skin sensitization experiments.[51−54]

At the concentration of 1 mM adduct formation was observed for the following compounds; 3,4-dihydroxybenzaldehyde (**22**), 2-methoxybenzaldehyde (**21**), 3,4-dimethoxybenzaldehyde, and salicylaldehyde (**2**). Benzaldehyde (**14**), isophthalic dicarboxyaldehyde (**5**), and piperonal (**7**) gave a total adduct percent of <5 (Table 2). The observation that 2-methoxybenzaldehyde (**21**) and 3,4-dimethoxybenzaldehyde were not active against *M. incognita* in in vitro experiments led us to hypothesize a different and unspecific mechanism of action rather than an effect on the external cuticle of nematodes.

Peptide experimental average mass value, determined by HPLC-ESI-MS analysis, was 1508.7 ± 0.3 Da, in agreement with the theoretical mass value of 1508.6 Da expected for the reduced form. Furthermore, HPLC-ESI-MS analysis revealed the presence of oxidized derivatives of the peptide generated by intra- or intermolecular disulfide bridges. The reaction products

Table 2. Reactivity of the Peptide with Different Aldehydes: Relative Percentage of Total Adducts ((Monomeric + Dimeric) × 100/(Monomeric + Dimeric + Free Peptide))[a]

compound	1 mM	5 mM	15 mM
2-methoxybenzaldehyde	74	77	NT
3,4-dihydroxybenzaldehyde	72	89	73
3,4-dimethoxybenzaldehyde	65	70	NT
salicylaldehyde	61	57	NT
piperonal	5	30	NT
isophthalic dicarboxaldehyde	1	2	0
benzaldehyde	0	0	6

[a]NT indicates not tested.

of the peptide with different aldehydes, analyzed by HPLC-ESI-MS, are reported in Table 3. The mass values were in agreement with (a) the formation of monomeric and dimeric adducts with the aldehydes; (b) the binding of one, two, or three aldehyde moieties to the peptide depending on the reactivity of the aldehyde; and (c) the presence of acetal linkages in the adducts (observed Δmass = mass value of aldheyde − 18 Da). Such adduct formation can be linked to the reactivity between the peptide and test chemical, whereas peptide depletion often indicates peptide dimerization or other oxidative processes.

As far as it concerns the different reactivities to the aldehydes, the peptide bound up to two 3,4-dihydroxybenzaldehyde (**22**) and 2-methoxybenzaldehyde (**21**) moieties in the monomeric adducts and up to three moieties in the dimeric adducts, but the peptide bound up to two salicylaldehyde (**2**), 3,4-dimethoxybenzaldehyde, and piperonal (**7**) moieties in both monomeric and dimeric derivatives and only one benzaldehyde moiety in both the monomeric and dimeric peptide derivatives. In the reaction with isophthalic dicarboxaldehyde the peptide generated only the monomeric 1:1 adduct.

Cysteine reduction/alkylation experiments showed that the reaction involved the cysteine residues. Indeed, in the monomeric adduct linking one salicylaldehyde (**2**) (or 3,4-dihydroxybenzaldehyde (**22**)) moiety, only two cysteines were alkylated, and in that one linking two 3,4-dihydroxybenzaldehyde (**22**) moieties only one cysteine was alkylated. The monomeric adduct was never observed with all three cysteine residues alkylated, confirming the involvement of the cysteine residues in the formation of thioacetal linkages with the aldehydes. Following the cysteine alkylation, the dimeric adducts linking one, two, or three aldehyde moieties showed four, two, or no alkylated cysteines, respectively. This finding suggests that the dimeric adducts are dithioacetals generated by the cross-linking of the aldehyde with two cysteine residues belonging to different peptide chains.

Table 3. Adducts Generated by the Reaction of MjCol3 Peptide with Various Aldehydes at Different Concentrations: Experimental and Theoretical Average Mass (Mav) Values (Da) of the Products of Reaction, Ratio of Peptide/Linked Aldehydes, and Its Relative Percentage[a]

				relative %		
aldehyde	reaction products exptl Mav (theor Mav)	attribution	peptide/aldehyde	1 mM	5 mM	15 mM
2-methoxybenzaldehyde	1624.7 ± 0.3 (1626.8)	monomeric adduct*	1:1	**74** = 12	**77** = 18	NT
	1744.3 ± 0.3 (1744.9)	monomeric adduct	1:2	0	2	
	3132.6 ± 0.6 (3135.4)	dimeric adduct*	2:1	42	27	
	3253.1 ± 0.6 (3253.5)	dimeric adduct	2:2	20	21	
	3371.4 ± 0.6 (3371.7)	dimeric adduct	2:3	0	9	
3,4-dihydroxybenzaldehyde	1626.7 ± 0.3 (1628.7)	monomeric adduct*	1:1	**72** = 5	**89** = 13	**73** = 12
	1749.2 ± 0.3 (1748.8)	monomeric adduct	1:2	0	0	2
	3135.4 ± 0.6 (3137.3)	dimeric adduct*	2:1	40	37	25
	3256.8 ± 0.6 (3257.4)	dimeric adduct*	2:2	27	35	31
	3378.4 ± 0.6 (3377.6)	dimeric adduct	2:3	0	4	3
3,4-dimethoxybenzaldehyde	1654.9 ± 0.3 (1656.8)	monomeric adduct*	1:1	**65** = 7	**70** = 6	NT
	3163.4 ± 0.6 (3165.4)	dimeric adduct*	2:1	44	42	
	3313.0 ± 0.6 (3313.6)	dimeric adduct	2:2	14	22	
salicylaldehyde	1610.7 ± 0.3 (1612.7)	monomeric adduct*	1:1	**61** = 9	**57** = 3	NT
	3118.7 ± 0.6 (3121.3)	dimeric adduct*	2:1	52	42	
	3222.9 ± 0.6 (3225.4)	dimeric adduct*	2:2	0	12	
piperonal	1640.6 ± 0.3 (1640.7)	monomeric adduct dimeric	1:1	**5** = 1	**30** = 4	NT
	3147.3 ± 0.6 (3149.3)	adduct*	2:1	4	26	
isophthalic dicarboxaldehyde	1624.1 ± 0.3 (1624.7)	monomeric adduct	1:1	0.7	1.6	0
benzaldehyde	1596.5 ± 0.3 (1596.7)	monomeric adduct	1:1	0	0	**6** = 1
	3105.6 ± 0.6 (3105.3)	dimeric adduct	2:1			5

[a]An asterisk (*) indicates oxidized derivatives (observed Δmass = −2 Da with respect to expected one).

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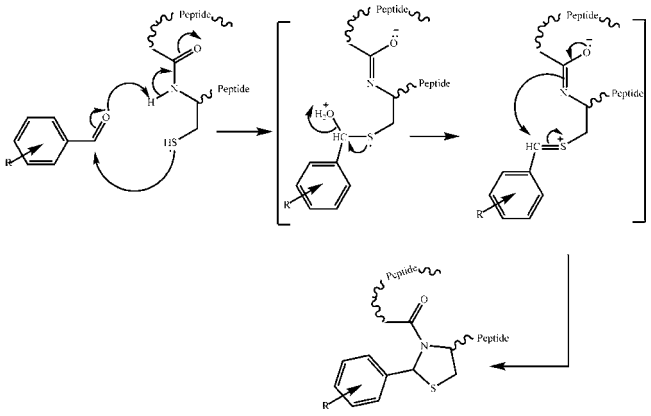
Moreover, quantitative analysis evidenced that the major peptide reactivity was toward 3,4-dihydroxybenzaldehyde (**22**), 3,4-dimethoxybenzaldehyde, and 2-methoxybenzaldehyde (**21**), followed by salicylaldehyde (**2**), piperonal (**7**), benzaldehyde (**14**), and isophthalic dicarboxaldehyde (**5**); these last generated the lowest quantity of adducts also at the higher concentration tested (15 mM).

A plausible explanation might be related to the fact that the reaction between the sulfur large-sized nucleophile and the carbonyl carbon atom can be considered as a soft−soft interaction, mainly orbital controlled. Thus, conversely to the acetalization process, which is charge-controlled, thioacetalization reaction is energetically favored when electron-rich carbonyl substrates are employed.[55] Besides, in relation to the high molecular orbital coefficient at the carbonyl carbon atom, the large orbital of the sulfur nucleophile may overlap efficiently, so satisfying the Bürgi−Dunitz trajectory.[56] A mechanism for monomer adduct formation is depicted in Scheme 3. A possible proton transfer to the oxyanionic form of the thiohemiacetal from the amide backbone and the subsequent formation of a (S,N) cyclic acetal is proposed.[57,58]

Scheme 3. Possible Reaction Mechanism for Monomer Adduct Formation



The relative abundances of total adducts generated are reported in Table 3. The major products of the reaction were the dimeric adducts. This might probably be due to the fact that the sulfur of the −SH residue is a better electron donor to an intermediate thiocarbenium ion than the nitrogen. The higher percentages of dimeric adducts were observed in presence of 5 mM 3,4-dihydroxybenzaldehyde (**22**) (76.0%), followed by 3,4-dimethoxybenzaldehyde (65%), 2-methoxybenzaldehyde (**21**) (57%), salicylaldehyde (**2**) (54%), and piperonal (**7**) (27%).

Unfortunately, it was not observed for the tested compounds a correlation between reactivity and potency because other factors, such us cuticle penetration or the activity against other biochemical targets, may be involved.

When we tested the toxicity of the V-ATPase inhibitor pyocyanin (**10**) against *M. incognita* J2, an EC_{50} at 24 h of 72 ± 25 mg/L was found. This toxicity against J2 was comparable with structurally similar compounds such as 2-pyridinecarboxaldehyde, indole-3-carboxyaldehyde, and pyrrole-2-carboxaldehyde (3.60 ± 1.16, 301 ± 33, and 392 ± 32 mg/L, respectively.[59] Consistent with previous works[15,59] we observed the J2 nematodes treated with pyocyanin (**10**) and tested aldehydes were paralyzed or died filled with liquid in a straight shape. Liegeois observed that *C. elegans* lacking larval expression of the V-ATPase genes often fill with fluid and die,[60] which mimics the phenotype observed by Nelsons when they ablated nematode excretory pore.[61] All of these facts suggest that the tested aromatic aldehydes probably act by inhibiting the nematode V-ATPase enzyme.

Moreover, Mahajan-Miklos et al. reported that pyocyanin (**10**) was responsible for the fast killing of the nematode *C. elegans* by producing reactive oxygen intermediates, and this effect was exacerbated by high levels of osmolarity.[62]

It is noteworthy that when ouabain, an inhibitor of the plasma membrane Na^+/K^+ ATPase, was tested against *M. incognita* J2, we did not observe any activity. Additionally, Kim et al. reported that *o*-hydroxybenzaldehydes such as 2-hydroxy-5-methoxybenzaldehyde and *o*-vanillin showed strong antifungal activity against filamentous fungi.[63] Moreover, these authors showed that when oxidative stress agents, such as *o*-hydroxybenzaldehyde, were applied to *sakA*Δ and *mpkC*Δ MAPK mutant strains of *A. fumigatus*, they became susceptible because the mutated MAPK system was incapable of launching a fully operational oxidative stress response.[63]

Although the mode of action of nematicidal aldehydes reported in this paper is not completely clarified and understood, we suppose that tested aldehydes may inhibit the V-ATPase enzymes. Further work should be done to assay nematode ATPase or proton translocation activities, and this requires an essentially intact enzyme complex that cannot be readily prepared in recombinant form.[64]

AUTHOR INFORMATION

Corresponding Author

*Phone: +39 070 675 8617. Fax: +39 070 675 8612. E-mail: caboni@unica.it.

Notes

The authors declare no competing financial interest.

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