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Potent Nematicidal Activity of Maleimide Derivatives on *Meloidogyne incognita*

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ABSTRACT: Different maleimide derivatives were synthesized and assayed for their in vitro activity on the soil inhabiting, plant-parasitic nematode *Meloidogyne incognita*, also known as root-knot nematode. The compounds maleimide, *N*-ethylmaleimide, *N*-isopropylmaleimide, and *N*-isobutylmaleimide showed the strongest nematicidal activity on the second stage juveniles of the root-knot nematode with $EC_{50/72h}$ values of 2.6 ± 1.3, 5.1 ± 3.4, 16.2 ± 5.4, and 19.0 ± 9.0 mg/L, respectively. We also determined the nematicidal activity of copper sulfate, finding an EC_{50} value of 48.6 ± 29.8 mg/L. When maleimide at 1 mg/L was tested in combination with copper sulfate at 50 mg/L, we observed 100% mortality of the nematodes. We performed a GC-MS metabolomics analysis after treating nematodes with maleimide at 8 mg/L for 24 h. This analysis revealed altered fatty acids and diglyceride metabolites such as oleic acid, palmitic acid, and 1-monopalmitin. Our results suggest that maleimide may be used as a new interesting building block for developing new nematicides in combination with copper salts.

KEYWORDS: *V-ATPase, metabolomics, oxidative stress, redox-active metals, nematicide*

INTRODUCTION

Nematodes are small soil worms; some of them are plant parasites and play a significant role in the susceptibility of the host plant to the attack by other pathogens.[1] Worldwide crop losses due to plant-parasitic nematode infections are estimated at USD 157 billion per year.[2] Root-knot nematodes (RKN), *Meloidogyne* spp., are widely distributed, polyphagous parasites infesting a wide range of host plants including horticultural crops such as cucumber, aubergine, melon, pepper, tomato, and fruit trees making them one of the major agricultural pests in the world.[3,4] Plant symptoms include formation of galls on root systems, due to hyperplasia and hypertrophy as reaction to the trophic activity of the nematode juveniles, retarded development and growth of hosts as well as reduced quality and quantity of the harvest. In the case of severe nematode attack, plants can eventually die. Although chemical nematicides are the most reliable means of controlling RKN, concerns for the environment have led to the progressive withdrawal of many synthetic nematicides and to the search of alternative strategies or novel and specific biochemical targets.[5] Recently, we reported on the nematicidal activity of arylhydrazones,[6] acetophenones, and chalcones on *Meloidogyne incognita*.[7] Current research is also focused on the potential use of natural plant extracts for the development of new nematicides.[8−14]

Modern toxicology employs new analytical methods to study toxicological effects. Metabolomics is a dynamic method of research that involves qualitative and quantitative measurements of metabolic responses in a living organism due to a genetic modification, external stimulus, and/or stressor. The field of metabolomics has grown rapidly over the past decade and has been proven to be a powerful omics methodology in predicting and explaining complex phenotypes in diverse biological systems.[15] When metabolomics was used, it revealed that imidacloprid induced metabolic changes at low and environmentally relevant concentrations in a nontarget species, leading to a novel mechanistic hypothesis.[16]

Maleimide (**1**) is a five-membered ring compound belonging to the cyclic imides class. Maleimide derivatives are emerging as potent pharmacophores showing different biological activities.[17,18] When investigated on *Escherichia coli* and *Staphylococcus aureus*, maleimides were approximately 30 times more active than succinimides.[19] When tested on *Sclerotinia sclorotiorum*, different synthesized maleimide derivatives showed an interesting fungicidal activity.[20] Moreover, by acting like thiol reagents, maleimides like *N*-ethylmaleimide and related *N*-arylcyclic imides show herbicidal activity.[21] At low concentrations (1−2 μM), *N*-ethylmaleimide (**2**), a covalently binding cysteinyl reagent,[22,23] is considered an inhibitor of the eukaryotic vacuolar (V-type) ATPases, while at higher concentrations (0.1−1 mM), it inhibits the P-type ATPases, F-type ATPases that are resistant to inhibition by **2**.[24,25] In the present study, starting with the hypothesis of the involvement of maleimide in the inhibition of the nematode V-ATPase activity, we prepared various maleimides and succinimides with different substituents and assessed their nematicidal activity in vitro. The synergistic activity of maleimide with copper and iron sulfates was also tested. Furthermore, we performed an in vitro GC-MS metabolomics analysis on nematodes, after their treatment with maleimide, by measuring the levels of ammonia excretion.

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MATERIALS AND METHODS

General Methods. Uncorrected melting points were measured on an SMP1 apparatus melting point apparatus (Stuart Scientific, Stone, UK). Proton NMR spectra were acquired on Inova 500 spectrometer (Varian, Palo Alto, CA). The chemical shifts are reported in parts per million (δ, ppm) downfield from tetramethylsilane (TMS) used as internal standard. Infrared spectra were obtained with a Vector 22 spectrophotometer (Bruker, Bremen, Germany). Purity of synthesized chemicals was determined by an elemental analyzer (Yanagimoto, Kyoto, Japan) and was greater than 95% when compared with the theoretical values. High resolution mass spectra of synthesized compounds were acquired using an Agilent LC-QTOF 6520 (Agilent Technologies, Palo Alto, CA). Thin layer chromatography (TLC) on E. Merck TLC plates coated with silica gel 60 F254 (0.25 mm layer thickness) was used to monitor reaction courses. TLC visualization was carried out using a UV lamp at 254 nm.

Materials. All synthetic precursors and solvents were purchased from Sigma-Aldrich (Milan, Italy). Maleimides and succinimides **1**, **2**, **3**,[26] **4**,[20] **5**,[27] **8**,[27] **9**, **10**,[27] **11**,[28] **12**,[29] **13**,[29] **15**,[30] **16**,[29] **17**,[31] **18**,[20] **19**,[31] **21**,[28] **24**,[29] **26**,[29] **27**,[32] and **28**[27] were commercially available or obtained as previously reported.

General Procedure for the Synthesis of Maleimides. A mixture of maleic anhydride (0.196 g, 2 mmol) and the appropriate amine (2 mmol) in CH_2Cl_2 (5 mL) was stirred at room temperature for 1 h. The solvent was removed in vacuo, and the residue, maleamic acid, was then dissolved in 3 mL of acetic anhydride. To this solution we added sodium acetate (0.10 g, 1.22 mmol), and the mixture was then heated for 2 h at 100 °C and cooled with water afterward. The aqueous solution was extracted with Et_2O (3 × 10 mL), and the organic layers were collected, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo to give N-substituted maleimides **2**−**8** and **11**−**28**.

1-(Heptan-2-yl)-1H-pyrrole-2,5-dione ***96***. Yield 82%, colorless oil. ^{1}H NMR (DMSO-d_6): δ 6.95 (s, 2H, CH), 4.00−4.03 (m, 1H, CH), 1.79−1.86 (m, 1H, CH_2), 1.52−1.59 (m, 1H, CH_2), 1.27 (d, J = 7.0 Hz, 3H, CH_3), 1.09−1.23 (m, 6H, CH_2), 0.81 (t, J = 7.0 Hz, 3H, CH_3). IR (neat) 1710, 1457, 1405, 1369 cm^{-1}. HRMS (ESI-TOF) (m/z) [M + H]$^+$ calcd for ($C_{11}H_{17}NO_2$) 196.1332, found 196.1335. Calcd: C, 67.66; H, 8.78; N, 7.17. Found: C, 67.67; H, 8.76; N, 7.18.

1-(6-Methylheptan-2-yl)-1H-pyrrole-2,5-dione ***(7)***. Yield 79%, colorless oil. ^{1}H NMR (DMSO-d_6): δ 6.95 (s, 2H, CH), 4.00−4.03 (m, 1H, CH), 1.78−1.84 (m, 1H, CH_2), 1.51−1.55 (m, 1H,CH_2) 1.42−1.47 (m, 1H, CH_2), 1.27 (d, J = 6.5 Hz, 3H, CH_3), 1.06−1.15 (m, 4H, CH_2), 0.80 (t, J = 7.0 Hz, 6H, CH_3). IR (neat) 1704, 1460, 1405, 1367 cm^{-1}. HRMS (ESI-TOF) (m/z) [M + H]$^+$ calcd for ($C_{12}H_{19}NO_2$) 210.1489, found 210.1487. Calcd: C, 68.87; H, 9.15; N, 6.69. Found: C, 68.89; H, 9.13; N, 6.70.

1-(4-Fluoro-3-nitrophenyl)-1H-pyrrole-2,5-dione ***(14)***. Yield 82%, mp 115−118 °C. ^{1}H NMR (DMSO-d_6): δ 7.24 (s, 2H, CH), 7.72−7.83 (m, 2H, Ar), 7.85 (s, 1H, Ar). IR (Nujol) 1714, 1594, 1538, 1503 cm^{-1}. HRMS (ESI-TOF) (m/z) [M + H]$^+$ calcd for ($C_{10}H_5FN_2O_4$) 237.0306, found 237.0302. Calcd: C, 50.86; H, 2.13; F, 8.04; N, 11.86. Found: C, 50.87; H, 2.15; F, 8.03; N, 11.88.

1-(4-(Methylthio)phenyl)-1H-pyrrole-2,5-dione ***(20)***. Yield 63%, mp 76−79 °C. ^{1}H NMR (DMSO-d_6): δ 7.31 (s, 2H, CH), 7.35 (d, J = 8.5 Hz, 2H, Ar), 7.26 (d, J = 8.5 Hz, 2H, Ar), 2.49 (s, 3H, CH_3). IR (Nujol) 1793, 1768, 1706, 1665 cm^{-1}. HRMS (ESI-TOF) (m/z) [M + H]$^+$ calcd for ($C_{11}H_9NO_2S$) 220.0427, found 220.0429. Calcd: C, 60.26; H, 4.14; N, 6.39; S, 14.62. Found: C, 60.25; H, 4.12; N, 6.36; S, 14.63.

1-(4-Ethoxyphenyl)-1H-pyrrole-2,5-dione ***(22)***. Yield 75%, mp 78−80 °C. ^{1}H NMR (DMSO-d_6): δ 7.38 (d, J = 8.5 Hz, 2H, Ar), 7.18 (s, 2H, CH), 6.98 (d, J = 8.5 Hz, 2H, Ar), 4.05 (q, J = 7.0 Hz, 2H, CH_2), 1.32 (t, J = 7.0, 3H, CH_3). IR (Nujol) 1793, 1763, 1713, 1666 cm^{-1}. HRMS (ESI-TOF) (m/z) [M + H]$^+$ calcd for ($C_{12}H_{11}NO_3$) 218.0811, found 218.0810. Calcd: C, 66.85; H, 5.10; N, 6.45. Found: C, 66.83; H, 5.12; N, 6.46.

1-(3-Chloro-4-methoxyphenyl)-1H-pyrrole-2,5-dione ***(23)***. Yield 56%, mp 132−135 °C. ^{1}H NMR (DMSO-d_6): δ 7.49 (s, 1H, Ar), 7.21 (d, J = 8.5 Hz, 1H, Ar), 7.16 (s, 2H, CH), 7.10 (d, J = 8.5 Hz, 1H, Ar), 3.88 (s, 3H, CH_3). IR (Nujol) 1770, 1712, 1685, 1560 cm^{-1}. HRMS (ESI-TOF) (m/z) [M + H]$^+$ calcd for ($C_{11}H_8ClNO_3$) 238.0265, found 238.0262. Calcd: C, 55.60; H, 3.39; Cl, 14.92; N, 5.82. Found: C, 55.62; H, 3.36; Cl, 14.90; N, 5.84.

1-(Naphthalen-2-yl)-1H-pyrrole-2,5-dione ***(25)***. Yield 72%, mp 105 °C. ^{1}H NMR (DMSO-d_6): δ 7.87−8.03 (m, 3H, Ar), 7.78 (s, 1H, Ar), 7.47−7.63 (m, 3H, Ar), 7.16 (s, 2H, CH). IR (Nujol) 1787, 1764, 1711, 1675 cm^{-1}. HRMS (ESI-TOF) (m/z) [M + H]$^+$ calcd for ($C_{14}H_9NO_2$) 224.0706, found 224.0705. Calcd: C, 75.33; H, 4.06; N, 6.27. Found: C, 75.31; H, 4.08; N, 6.30.

1-Benzylpyrrolidine-2,5-dione ***(10)***. A mixture of benzylamine (0.21 g, 2 mmol), succinic anhydride (0.2 g, 2 mmol), and anhydrous triethylamine (1 mL, 7.2 mmol) in 25 mL of toluene was refluxed with a Dean−Stark apparatus for 36 h. The reaction mixture was cooled then concentrated under reduced pressure. The residue was dissolved in ethyl acetate (25 mL) and washed with saturated aqueous $NaHCO_3$ solution (3 × 10 mL) and then with water (3 × 10 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under vacuum to give the title compound in 80% yield. Analytical and spectroscopic data were in accordance with literature values.[27]

Nematode Population. A *M. incognita*[33] population was reared for two months at 25 ± 2 °C on susceptible tomato plants (*Solanum lycopersicum* L.; cv. Rutgers) in a greenhouse located in Bari, Italy. Infested plants were uprooted, and roots showing galls and egg masses were washed to remove soil. The roots were then cut into 2 cm pieces and the egg masses hand-picked. Batches of 20 egg masses of similar size (averaging 20000 eggs) were placed on 2 cm diameter sieves (215 μm), and each sieve was put in a 3.5 cm diameter plastic Petri dish; we then added 3 mL of distilled water (natural hatching agent for *Meloidogyne* spp.), sufficient to cover the egg masses, to allow the eggs to hatch. The dishes were incubated in a growth cabinet at 25 °C.[34] All second-stage juveniles (J2) which hatched in the first 3 d were eliminated, and only the J2s hatched after 24 h or more were collected and used to verify the in vitro nematicidal effect of the selected maleimides.

Nematicidal Assay. Maleimides and succinimides were tested for the nematicidal activity on the second stage juveniles and the corresponding EC_{50} values were calculated. Stock solutions of synthetic compounds were prepared using water, methanol, or DMSO as solvent, whereas working solutions were made by dilution with tap water. The final concentrations of the organic solvent in each well never exceeded 1% because preliminary experiments showed that the motility of nematodes exposed to those concentrations was similar to the motility of the controls. Tap water was used with the controls. Treatments were performed in 96-well plates using 30 juveniles in each replication. To avoid solvent evaporation, plates were covered and kept in the dark at 28 °C. After treatment, juveniles were moved to plain water and grouped into two distinct categories, motile or immotile, with the use of an inverted microscope at 40× after pricking the juvenile body with a needle. Nematodes that never regained movement after being moved to tap water and pricked were considered to be dead. Six replications were made, and the experiment repeated at least twice.

Synergistic activity of maleimide with metal salts was also studied. By assuming the linearity of the nematicidal assay, we used the method reviewed by Berenbaum.[35] The combination of two agents A and B is termed (d_a and d_b), where d_a and d_b are the concentrations of A and B, respectively. The effect is treated as a mathematical function E; thus $E(d_a,d_b)$ is the combined effect and $E(d_a)$ and $E(d_b)$ are the effects of the individual agents A and B, respectively. Zero interactive combination is observed when the summation of the effects of the individual agents is not significantly different from the combination effect, i.e., $E(d_a,d_b) = E(d_a) + E(d_b)$. If the combination effect is smaller or greater than the summation of the individual agents effects, the combination is antagonistic or synergistic, respectively. The comparison was made at two concentration levels, of maleimide and **1**, 1 and 2 mg/L, in combination with copper and iron sulfates at different concentration levels, 50 and 75 and 125 and 200 mg/L, respectively. The experiment was conducted at 24 and 72 h and repeated twice. The

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sum of dead J2s obtained from bioassays performed using solutions of each test compound separately (expected) was compared with the number of dead J2s observed and caused by immersion of *M. incognita* J2 in the respective combinations.[8]

Sample Extraction. For the metabolomics analysis, nematode polar metabolites extraction was performed after treating 300 juveniles of *M. incognita* J2 in vitro with 8 mg/L of maleimide (**1**) in a total volume of 200 μL. A negative control consisting of nematodes immersed in tap water was prepared. After 24 h, test solutions were transferred to 1.5 mL Eppendorf tubes and ultrasonicated with a Vibracell cell disruptor (Labotal Scientific Equipment, Abu Ghosh, Israel) for the nematode cuticle lysis. The ultrasonication was performed twice with 20 s pulses at 60% amplitude (130 W, 20 kHz). Finally, 800 μL of *tert*-butyl methyl ether were added to the samples, which were then vortexed for 1 min. After centrifugation at 18000 rpm at 25 °C for 15 min, the supernatant liquid was removed and dried overnight in glass vials. Extracts were submitted to a derivatization step for GC-MS analysis using a solution of methoxamine chloride dissolved in pyridine (10 mg/mL). Then 80 μL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) were added after 17 h from the treatment. After 1 h, we added 50 μL of hexane containing the internal standard 2,2,3,3-d_4-succinic acid (2 mg/L). Eight replications were made for every sample. The chromatographic separation was performed on a Agilent 6850 gas chromatograph coupled with a mass detector 5973 and a 7683B series injector autosampler (Agilent Technologies, Palo Alto, CA). The resulting data was analyzed using MSD ChemStation. The column was a 30 m × 0.25 mm, 0.25 μm DB5-MS (Agilent Technologies, Palo Alto, CA), and the injector temperature was set at 250 °C. The oven temperature program was as follows: from 50 to 230 °C at 5 °C/min for 36 min and kept at this temperature for 2 min. Helium was the carrier gas, at a constant flow rate of 1 mL/min, and 1 μL of the sample was injected in the splitless mode. The detector settings were as follows: ionization voltage, 70 eV; scan rate, 2.91 scan/s; mass range, 50−550; transfer line, 230 °C. Metabolites were identified by (a) comparison of their relative retention times and mass fragmentation with those of authentic standards and (b) computer matching against NIST98, as well as retention indices as calculated according to Kovats, for the series of alkanes C9−C24.

Statistical Analysis. According to the Schneider Orellis formula, we corrected the percentages of dead juveniles by eliminating the natural death control samples (5% of total number of J2):[36]

$$\text{corrected \%} = ((\text{mortality \% in treatment} - \text{mortality \% in control})/(100 - \text{mortality \% in control})) \times 100 \quad (1)$$

and they were analyzed (ANOVA) after being combined over time and means were averaged over experiments. Afterward, corrected percentages of dead J2s were submitted to a nonlinear regression analysis according to Seefeldt et al.[37]

eq 2

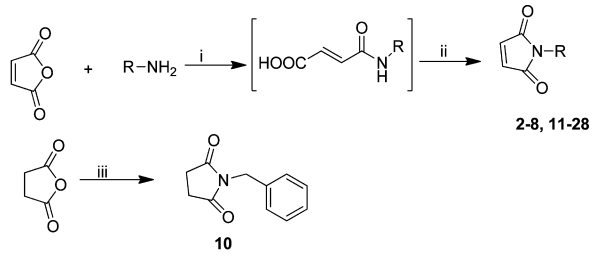
$$Y = C + \frac{D - C}{1 + e^{b \log x - \log EC_{50}}} \quad (2)$$

where the EC_{50} is the test compound concentration required for 50% death/immotility of nematodes compared to natural death/immobility in the control, C = the lower limit, D = the upper limit, and b = the slope at the EC_{50}. In the regression equation, the test compounds concentration (% w/v) was the independent variable (x) and the immotile J2 (percentage increase over water control) was the dependent variable (y). The mean value of the six replicates was used to the EC_{50} evaluation.

Multivariate Analysis. From the data obtained through the GC-MS analysis, a matrix of 16 × 28 data composed of the samples analyzed (eight controls and eight treated samples) and from the areas of the chromatographic peaks (28 variables) was built. When a variable showed an abnormal distribution, it was canceled using a logarithmic transformation validated from the Skewness test; statistical analysis was performed by the SIMCA software, version 14.0 (Umetrics, Umea, Sweden). Prior to analysis, data arrays were subjected to centering through the centering unit variance. The matrices obtained were subjected to multivariate analysis. Using software SIMCA-P, the following analyses were made: principal components analysis (PCA) and a discriminant regression method (PLS-DA) and its orthogonal extension (OPLS-DA). The quality of the model has been validated on the basis of the parameters R^2X (change in X explained by the model), R^2Y (the total of Y explained), and Q^2 (sum parameter in cross-validation).[38] A scatter plot of possible discriminant metabolites was obtained using an S-plot, which combines covariance and correlation loading profiles.

RESULTS AND DISCUSSION

We report for the first time the nematicidal activity of maleimide derivatives. A series of maleimide and succinimide derivatives were synthesized through the one-pot two-step procedure described in Figure 1, and their nematicidal activity



compd	R	compd	R
2	Et	**17**	3,4-diCl-phenyl
3	i-Pr	**18**	3,5-diCl-phenyl
4	i-Bu	**19**	2,3-diCl-phenyl
5	n-Bu	**20**	4-SMe-phenyl
6	1-Me-hexyl	**21**	4-OMe-phenyl
7	1,5-diMe-hexyl	**22**	4-OEt-phenyl
8	benzyl	**23**	3-Cl, 4-OMe-phenyl
11	4-NO_2-phenyl	**24**	4-iPr-phenyl
12	4-Cl-phenyl	**25**	2-naphthyl
13	4-F-phenyl	**26**	4-OH-phenyl
14	4-F, 3-NO_2-phenyl	**27**	4-COOH-phenyl
15	4-CF_3-phenyl	**28**	4-COOEt-phenyl
16	2-Cl-phenyl		

Figure 1. Synthetic route to maleimides. Reagents and conditions: (i) CH_2Cl_2, 1 h, rt; (ii) $(CH_3CO)_2O$, $NaOCOCH_3$, 2 h, 100 °C; (iii) TEA, toluene, 36 h, reflux.

was tested in vitro on second stage juveniles of the root-knot nematode *M. incognita*. Initially, we explored the effectiveness of the maleimide derivatives testing each compound at the concentration of 100 mg/L after 72 h treatments (Table 1). Compounds showing a paralyzing activity greater than 90% were then subjected to $EC_{50/72h}$ determination (Table 2). For comparison, abamectin and fosthiazate were used as chemical control, with $EC_{50/72h}$ of 2.1 ± 1.2 and 0.6 ± 0.2 mg/L, respectively.

The maleimide, **1**, showed an $EC_{50/72h}$ of 2.6 ± 1.3 mg/L while the succinimide, **9**, was not active. The *N*-ethylmaleimide, **2**, *N*-isopropylmaleimide, **3**, and *N*-isobutylmaleimide, **4**, showed 100% mortality in the primary screening experiment and $EC_{50/72h}$ values in the 2.6−19 mg/L range (5.1 ± 3.4, 5.1 ± 3.4, and 19.0 ± 9.0, respectively). On the contrary, reduced activity is produced by the *n*-butyl derivative, **5**, as well as by the introduction of a long chain as in compounds, **6**−**8**. Among *N*-arylmaleimides, the 4-nitrophenyl, **11**, the 4-chlorophenyl, **12**, and the 4-carboxyethylphenyl, **28**, were the most effective ($EC_{50/72h}$ = 23.8 ± 3.0, 53.4 ± 3.0, and 60.9 ± 20.6,

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Table 1. Percentage Nematicidal Activity of Tested Maleimide and Succinimide Derivatives at 100 mg/L after 72 h (n = 6)

compd	mortality (%) ± SD	compd	mortality (%) ± SD
maleimide (**1**)	100 ± 0.0	**15**	6.9 ± 5.0
2	100 ± 0.0	**16**	12.0 ± 4.0
3	100 ± 0.0	**17**	7.7 ± 3.9
4	100 ± 0.0	**18**	11.0 ± 9.7
5	28.0 ± 8.2	**19**	8.9 ± 3.0
6	35.0 ± 6.6	**20**	7.5 ± 2.9
7	40.0 ± 7.1	**21**	18.8 ± 4.8
8	15.7 ± 9.7	**22**	20.6 ± 10.0
succimide (**9**)	NA[a]	**23**	11.0 ± 5.4
10	NA[a]	**24**	22.8 ± 7.5
11	100 ± 0.0	**25**	11.3 ± 2.5
12	100 ± 0.0	**26**	40.5 ± 8.0
13	10.8 ± 2.3	**27**	NA[a]
14	6.4 ± 0.8	**28**	92.3 ± 3.3

[a]NA = not active.

Table 2. $EC_{50/72h}$ of the Most Active Maleimide Derivatives and Copper and Iron Sulfate Solutions

compd	$EC_{50/72h}$ ± SD (mg/L)
1	2.6 ± 1.3
2	5.15.1 ± 3.4
3	16.2 ± 5.4
4	19.0 ± 9.0
11	23.8 ± 3.0
12	53.4 ± 3.0
28	60.9 ± 20.6
copper sulfate	48.6 ± 29.8
iron sulfate	126 ± 48
fosthiazate	0.6 ± 0.2
abamectin	2.1 ± 1.2

respectively). The shift of chlorine to the 2-position, **16**, or the introduction of a further chlorine atom, as in maleimides, **17**–**19**, led to a reduction in activity. The nematicidal activity dropped to 6–20% of mortality after 72 h at 100 mg/L when a 4-nitro or a 4-chlorine group was replaced by a fluorine, **13**, trifluoromethyl, **15**, ether, **21**–**22**, or thioether, **20**, groups. Furthermore, the introduction of a nitro, **14**, or a chlorine, **23**, group on the aryl ring failed to enhance the nematicidal activity of maleimides, **13** and **21**, respectively. The replacement of the ester moiety of maleimide, **28**, with a carboxyl group produced the inactive derivative, **27**. The introduction in the same position of a hydroxyl group produced maleimide **26**, which also showed a reduced nematicidal activity.

Metabolomics is one of the latest omics sciences which allows the unsupervised or supervised determination of low molecular mass metabolites in biological samples. Recently, we reported on the use of metabolomics in detecting upregulated or downregulated metabolites in *M. incognita* treated with different nematicidal arylhydrazones.[6] Using a GC-MS metabolomics approach, we detected different endogenous metabolites such as carbohydrates, amino acids, fatty acids, and monoacylglycerols. Statistical significant polar metabolites were studied using the software SIMCA-P (Umetrics, Umea, Sweden, ver. 14.0.0.1359). The first step was to perform a PCA to examine interrelation between groups, clustering, and outlier diagnostics among the samples. In the second step, a PLS-DA was performed to maximize the difference of metabolic profiles between treated and control samples and allowing metabolite recognition. The final step of the statistical analysis was to perform a supervised OPLS-DA with the goal to separate samples in two clusters and identify potential biomarkers among nematodes treated with maleimide at 8 mg/L for 24 h and the control group. Statistical parameters for the OPLS-DA model were: Q^2 = 0.63, R^2X = 0.60, and R^2Y = 0.80 (Figure 2). The permutation test parameters relative to PLS-DA were: R^2 = 0.61 and Q^2 = −0.47, respectively. Upregulated metabolites for *M. incognita* nematodes treated with maleimide were in decreasing order oleic acid and palmitic acid, while the downregulated metabolite was 1-monopalmitin. Altered levels of fatty acids are probably related to the monounsaturated fatty acids which are essential components of membrane and storage lipids. Their synthesis depends on the conversion of saturated fatty acids to unsaturated fatty acids by Δ^9-desaturases.[39] Biosynthesis of oleic acid starting from palmitic acid in our inhibition experiment with maleimide may be a scavenger strategy of the nematodes to cope with reactive oxygen species.[40,41] Considering the small pool of metabolites identified in this work, further metabolomic studies, using different analytical platforms and different extraction procedures, are needed to generate a full nematode metabolome and contribute with a mechanistic assessment.

Moreover, considering that *vma* yeast mutants, lacking V-ATPase subunits, are hypersensitive to multiple forms of oxidative stress,[42] suggesting an antioxidant role of V-ATPase, we treated nematodes with two redox active metals, i.e., copper and iron as sulfates known to be able to produce superoxide radicals.[43] In our experimental conditions, both metal salts were highly toxic to *M. incognita*, with EC_{50}s of 48.6 ± 29.8 and 126 ± 48 mg/L for copper and iron sulfate, respectively (Table 2).

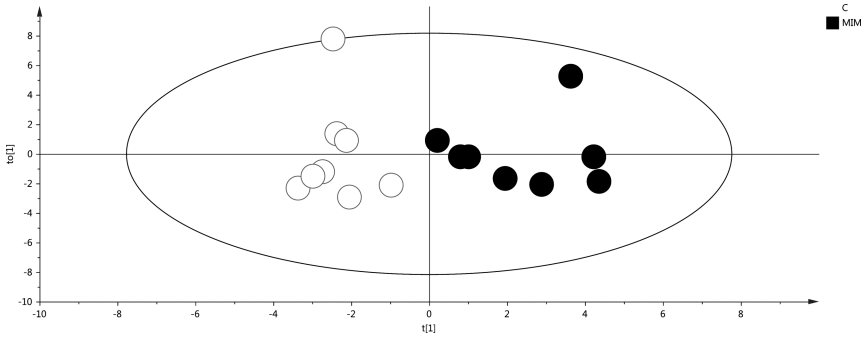

Figure 2. Score plot of OPLS-DA of nematodes treated with maleimide **1** (black circles) vs controls (white circles).

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Table 3. Synergistic Activity of Maleimide 1 in Combination with Cu^{2+} and Fe^{3+} after 24 and 72 h

		mortality (%) ± SD			
		24 h		72 h	
combination	concentration (mg/L)	expected	observed	expected	observed
1/Cu^{2+}	1/50	2.2 ± 2.6	84.6 ± 8.7	8.8 ± 2.5	100
	1/75	25.7 ± 5.5	61.7 ± 4.2	100	100
	2/50	11.4 ± 9.5	93.5 ± 5.8	100	100
	2/75	35.2 ± 9.0	100	100	100
1/Fe^{3+}	1/125	3.6 ± 2.7	35.1 ± 6.5	7.2 ± 2.1	78.2 ± 3.7
	1/200	34.6 ± 5.9	33.2 ± 7.0	89.3 ± 7.3	93.9 ± 5.2
	2/125	12.9 ± 6.2	95.6 ± 3.5	30.7 ± 3.4	100
	2/200	100	100	90.0 ± 5.6	100

Furthermore, we measured the synergistic activity of maleimide, **1**, with copper and iron sulfate. After 72 h, we observed a synergistic nematicidal activity with a strength increased by a factor of 10 (Table 3). Consistent with our previous experiments with (*E*)-1-((3-methylthiophen-2-yl)methylene)-2-phenylhydrazine,[6] when nematodes where treated with the V-ATPase inhibitor **1** at 2 and 6 h, we measured a 3.5-fold increase in the excretion of ammonium (Table 4). On the other hand, levels of sodium, potassium, magnesium, and calcium were not altered. Thus, assuming that maleimide derivatives were able to inhibit the nematode V-ATPase at low EC_{50} values and considering that their strength is greatly augmented when used in combination with redox metals such as copper and iron, these compounds may potentially be used to develop new active ingredients with nematicidal properties or be used in the control of root knot nematodes in the field.

Table 4. Excretion Levels of Some Cations when Nematodes were Treated with Maleimide 1 at 8 mg/L after 2 and 6 h

		concentration (mg/L)	
cation	time (h)	**1**	control
Na^+	2	2.9 ± 0.3	2.6 ± 0.1
	6	2.5 ± 0.1	2.6 ± 0.2
NH_4^+	2	0.057 ± 0.007	0.016 ± 0.004
	6	0.076 ± 0.012	0.020 ± 0.003
K^+	2	1.1 ± 0.5	0.7 ± 0.1
	6	0.6 ± 0.0	0.6 ± 0.1
Mg^{2+}	2	1.9 ± 0.1	1,9 ± 0.1
	6	1.8 ± 0.2	1,7 ± 0.1
Ca^{2+}	2	6.9 ± 0.8	7.0 ± 0.5
	6	6.2 ± 0.5	6.7 ± 0.3

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Notes

The authors declare no competing financial interest.

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