JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

Cite This: J. Agric. Food Chem. 2019, 67, 10947−10953

pubs.acs.org/JAFC

Antiaflatoxigenic Thiosemicarbazones as Crop-Protective Agents: A Cytotoxic and Genotoxic Study

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Supporting Information

ABSTRACT: Aflatoxins are secondary fungal metabolites that can contaminate feed and food. They are a cause of growing concern worldwide, because they are potent carcinogenic agents. Thiosemicarbazones are molecules that possess interesting antiaflatoxigenic properties, but in order to use them as crop-protective agents, their cytotoxic and genotoxic profiles must first be assessed. In this paper, a group of thiosemicarbazones and a copper complex are reported as compounds able to antagonize aflatoxin biosynthesis, fungal growth, and sclerotia biogenesis in *Aspergillus flavus*. The two most interesting thiosemicarbazones found were noncytotoxic on several cell lines (CRL1790, Hs27, HFL1, and U937), and therefore, they were submitted to additional analysis of mutagenicity and genotoxicity on bacteria, plants, and human cells. No mutagenic activity was observed in bacteria, whereas genotoxic activity was revealed by the Alkaline Comet Assay on U937 cells and by the test of chromosomal aberrations in *Allium cepa*.

KEYWORDS: *aflatoxin, thiosemicarbazone, copper complexes, antiaflatoxigenic compounds, crop-protective agents*

INTRODUCTION

Mycotoxins, especially aflatoxins (AFs), are considered the most widespread cause of food contamination and spoilage worldwide.[1,2] These secondary fungal metabolites possess profiles of severe toxicity and carcinogenicity and can have teratogenic, carcinogenic, neurotoxic, and immunosuppressive effects.[3] The most common producers of mycotoxins are fungi belonging to the *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* genera, which grow especially in hot and humid climates. They can contaminate a large variety of important agricultural crops, like corn, peanuts, wheat, and rice, causing important economic losses and representing a risk for animal and human health.[4]

Up to now, the most common way to fight fungal diffusion and consequent crop contamination by mycotoxins has been the use of pesticides, but this has the consequence of generating noxious long-term residues in food and in the environment.[5] Moreover, the problem of resistant pest strains is an emerging global concern. All these considerations push toward the development of novel plant-protection products able to both minimize the prolonged use of synthetic fungicides and block the production of AFs. We have recently found a series of molecules, belonging to the thiosemicarbazone class, with very promising antifungal and antiaflatoxigenic profiles.[6−9] Our investigations highlighted that modifications on the backbone of the thiosemicarbazone scaffold provides a means to tune the activity against the two major genera of mycotoxigenic fungi that contaminate cereals, *Fusarium* and *Aspergillus*, and that metal chelation could improve the activity. By using these thiosemicarbazones and their copper complexes, it is in fact possible to obtain relevant antiaflatoxigenicity in concert with moderate fungistatic activity. This behavior has the advantage of avoiding problems related to undesired modifications of the composition of the microbiota in the environment.

The analysis of the previous results indicates that the presence of the C═S group is essential to the antiaflatoxigenic activity (the corresponding semicarbazones are inactive).[8] In addition, the antiaflatoxigenic activity depends deeply on the substituents on the phenyl ring, and it is increased by an increase in the lipophilicity of the thiosemicarbazone.[9] This last observation could be obviously associated with the ability of the molecule to cross the cell membrane.[10,11] As an extension of this research, we here present a study on phenyl and cyclohexyl thiosemicarbazones **1**−**4** (Figure 1).

These compounds have been tested for their ability to inhibit sclerotia biogenesis and AF biosynthesis in *Aspergillus flavus*. We also synthesized compound **5**, the copper complex of **1**, because previous results[12] suggested that metal chelation can lead to improved AF inhibition profiles and that in particular copper compounds are good candidates for inhibiting mold and bacteria growth and aflatoxin production.

Received: March 22, 2019
Revised: September 3, 2019
Accepted: September 9, 2019
Published: September 9, 2019

DOI: 10.1021/acs.jafc.9b01814
J. Agric. Food Chem. 2019, 67, 10947−10953

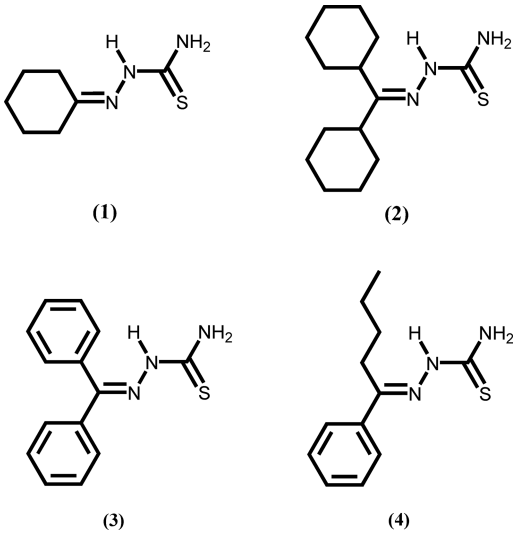

Figure 1. Schematic representation of thiosemicarbazones **1**−**4**.

In view of potential agronomical applications, it is important to assess the potential genotoxic risks to the environment and human health from the crop-protective agents. We have therefore investigated the cyto- and genotoxic effects of the most active compounds, **3** and **4**, on healthy human cells and in particular on human cell lines coming from tissues that can come into contact with chemicals (gastrointestinal tract, pulmonary epithelium, and epidermis). Finally, the toxic and genotoxic effects of **3** and **4** were investigated on bacteria and plant cells.

MATERIALS AND METHODS

The following commercial products were used: chemicals (Sigma-Aldrich Srl, Milano, Italy); Ham's Nutrient Mixture F-12 and fetal bovine serum (FBS) (EuroClone s.p.a., Milano, Italy); HFL1 (ATCC, CCL-153), CRL 1790 (ATCC, CCD 841 CoN), and Hs27 (ATCC, CRL1634) (American Type Culture Collection, ATCC, Manassas, VA); U937 cells (American Tissue Culture Collection, Rockville, MD); CellTiter96 AQ_{ueous}One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI); and Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 (Lonza Group Ltd., Basel, Switzerland).

Chemistry. The purities of the compounds were ≥95%, as determined by elemental analysis. Technical details about the NMR, ATR-IR, ESI-MS, and ICP analyses are reported elsewhere.[9]

The synthesis of **1**−**4** (Figure 1) was performed by using a slightly modified literature procedure.[9]

*Cyclohexanone Thiosemicarbazone (**1**).* Thiosemicarbazide (3.30 mmol) was dissolved in refluxing ethanol. Cyclohexanone (1.2 equiv, 3.96 mmol) and a few drops of glacial acetic acid were added to the solution, and the mixture was reacted for 24 h. The solvent was partially evaporated and a precipitate was isolated by filtration. The solid was recrystallized from ethanol to give a yellow solid. Yield: 31%, mp: 170−171 °C. IR (ATR, cm^{-1}): 3374 $\nu(NH_2)$; 3205, 3143 ν(NH); 2940 ν(CH); 1582 ν(C═N); 1073, 1035, 831 ν(C═S). MS-EI: m/z 171.1 (100, M^+). ^{1}H NMR (DMSO-d_6, 25 °C, ppm), δ: 10.12 (s, 1H, NH), 7.93(s, 1H, NH_2), 7.50 (s, 1H, NH_2), 2.40 (t, 2H, $H_{cyclohexyl}$), 2.23 (t, 2H, $H_{cyclohexyl}$), 1.63−1.56 (m, 6H, $H_{cyclohexyl}$).

*Dicyclohexylketone Thiosemicarbazone (**2**).* Thiosemicarbazide (3.30 mmol) was dissolved in refluxing ethanol. Dicyclohexylketone (1.2 equiv, 3.96 mmol) and some drops of glacial acetic acid were added, and the mixture was reacted for 50 h. The solvent was removed by vacuum, and the solid residue was purified by a chromatographic column (hexane/ethyl acetate, 7:3) to give a yellow solid. Yield: 15%, mp: 169−170 °C. IR (ATR, cm^{-1}): 3430 $\nu(NH_2)$; 3276, 3230, 3140 ν(NH); 2921 ν(CH); 1693 ν(C═N); 1081, 843 ν(C═S). MS-EI: m/z 255.3 (80, M^+). ^{1}H NMR (DMSO-d_6, 25 °C, ppm), δ: 10.21 (s, 1H, NH), 8.03 (s, 1H, NH_2), 7.41 (s, 1H, NH_2), 3.03 (t, 1H, HC-C═N), 2.25 (t, 1H, HC−C═N), 1.69−1.15 (20H, $CH_{cyclohexyl}$).

*Benzophenone Thiosemicarbazone (**3**).* Thiosemicarbazide (3.3 mmol) was dissolved in refluxing ethanol. Benzophenone (1.2 equiv, 3.96 mmol) and some drops of glacial acetic acid were added; then the mixture was reacted for 36 h. After concentration by vacuum, the precipitate was isolated by filtration and recrystallized from ethanol to give a pale orange powder. Yield: 15%, mp: 172−173 °C. IR (ATR, cm^{-1}): 3430 $\nu(NH_2)$; 3276, 3236, 3141 ν(NH); 2922 ν(CH); 1693 ν(C═N); 1081, 844 ν(C═S). MS-EI: m/z= 235.3 (80, M^+). ^{1}H NMR (DMSO-d_6, 25 °C, ppm), δ: 8.66 (s, 1H, NH), 8.40 (d, 2H, NH_2), 7.76−7.65 (m, 6H, CH_{arom}), 7.42−7.34 (6H, CH_{arom}).

*Valerophenone Thiosemicarbazone (**4**).* Thiosemicarbazide (3.3 mmol) was dissolved in refluxing ethanol. Valerophenone (butylphenylketone) (1.2 equiv, 3.96 mmol) and some drops of glacial acetic acid were added to the solution, which was reacted for 40 h. Solvent was removed by vacuum; the solid was then purified by chromatography (CH_2Cl_2/CH_3OH, 9:1). Yield: 23%. IR (ATR, cm^{-1}): 3378 $\nu(NH_2)$; 3226, 3149 ν(NH); 2957, 2929 ν(CH); 1588 ν(C═N); 1066, 847 ν(C═S). MS-EI: m/z 267.5 (100, M^+). ^{1}H NMR (DMSO-d_6, 25 °C, ppm), δ: 10.34 (s, 1H, NH), 8.24 (s, 1H, NH), 8.90−7.88 (3H, NH + CH_{arom}), 7.38−7.39 (3H, CH_{arom}), 2.87 (t, 2H, H_2C−C═N), 1.37 (t, 4H, $CH_{cyclohexyl}$), 0.88 (t, 3H, $CH_{cyclohexyl}$).

*$Cu_2(HL)(L)_2$ (**5**).* Cyclohexanone thiosemicarbazone is abbreviated as HL. The solution obtained by mixing under N_2 100 mg (2 equiv) of **1** in 10 mL of degassed methanol and 1 equiv of $CuCl_2{\cdot}2H_2O$ in 5 mL of the same solvent was stirred at room temperature. After 4 h, it was cooled, and the precipitate was filtered off and washed with cold diethyl ether. Green powder. Yield: 34%. IR (ATR, cm^{-1}): 3436 $\nu(NH_2)$; 3248, 3165 ν(NH); 2932, 2856 ν(CH); 1604 ν(C═N); 1034 ν(C═S).^{1}H NMR (DMSO-d_6, 25 °C), δ: 10.52 (s, 1H, NH); 7.64, 8.47 (2s, br, 1H+1H, NH_2); 2.60 (s, br, $CH_{cyclohexyl}$); 1.58−1.69 (m, br, $CH_{cyclohexyl}$). ESI-MS (CH_3OH): m/z = 701, $[Cu_2(HL)_2L + H]^+$, 5%; 405, $[Cu(HL)L + H]^+$, 100%; 234, $[Cu(HL)]^+$, 50%. ICP: Cu found 20.3 ± 0.7%, calcd for $Cu_2(L)_2(HL)$: 19.8%.

***Aspergillus flavus* Strains.** Two strains were used: the toxigenic strain CR10 and the atoxigenic strain TOϕ, isolated from corn kernels in the Po Valley.[6]

Effect on Aflatoxin Accumulation. Aflatoxin accumulation in a coconut milk derived medium (CCM) was determined by the high throughput procedure described in our previous works.[9,13,14]

Effects on Fungal Growth and on Sclerotia Biogenesis. Once the AF accumulation rate was assessed, mycelia from single wells were recovered at the sixth day of incubation, slightly dried on paper towels, and weighed. Biomass measures were then converted to percentage inhibition with respect to the controls (DMSO-treated cultures).

A 5 μL aliquot of aflatoxigenic strain CR10 spore suspension (approximately 10^6 conidia/mL) was point-inoculated in the center of a Petri dish (Ø = 5 cm) filled with Czapek Dox Agar (CZA) medium with 100 μM thiosemicarbazones. Control plates were added with 1% (v/v) DMSO. Plates were replicated in triplicate and incubated at 30°C in darkness; after 2 weeks of incubation, sclerotia were manually scraped from the colony surface and washed with a 70% ethanol solution to completely remove conidia and then dried for 3 days at 60 °C. Dry weights were assessed. The thiosemicarbazone inhibition rate on sclerotia production was expressed as a percentage with respect to the control. Plates were inoculated in triplicate.

One-way analysis of variance (Past 3.x software) was used for data analyses. Tukey's test was applied to the data relative to mycelial growth, aflatoxin accumulation, and sclerotia production; differences were regarded as significant if $p < 0.05$.

Cytotoxicity. The biological activities of the molecules with antifungal potential were assessed on normal cells: human fibroblast cell line Hs27 (ATCC, CRL1634), human lung epithelial cell line HFL1 (ATCC, CCL-153), and human colon epithelial cell line

DOI: 10.1021/acs.jafc.9b01814
J. Agric. Food Chem. 2019, 67, 10947−10953

Crl1790 (ATCC, CCD 841 CoN). These cell lines were chosen to investigate the exposure risks, because they are attributable to human tissues that could be involved in interactions with xenobiotics.[7] We also performed toxicological assays on human histiocytic lymphoma cell line U937 (ATCC, CRL-3253) cultured in RPMI-1640. Hs27 and CRL1790 were cultured in DMEM. HFL1 was cultured in Ham's Nutrient Mixture F-12. All media were supplemented with 10% (v/v) FBS, 1% penicillin (100 U/mL)/streptomycin (100 μg/mL), and 1% L-glutamine (2 mM). Flasks and plates were maintained at 37 °C and 5% CO_2 in a humidified atmosphere. Culture medium was refreshed every two or three days during subculturing. Hs27, CRL1790, and HFL1 cells were used between passage numbers 5 and 20. MTS assays (CellTiter96 AQ_{ueous} One Solution Cell Proliferation Assay) were performed to identify the antiproliferative effects of the compounds. Briefly, 100 μL of a suspension of cells in exponential growth (5×10^4 cells/mL in complete medium, without phenol red, supplemented with 5% FBS) were added to 96-well plates 24 h before treatment. Plates were incubated at 37 °C in a humidified 5% CO_2 incubator. After this recovery period, increasing concentrations of compounds (0.5, 1.0, 5.0, 10.0, 50.0, and 100.0 μM) were added to the medium, and the cells were left exposed for 24, 48, or 72 h. The negative control was represented by 100 μM DMSO. After the treatment period, 20 μL of MTS reagent was added to each well. At the end of the exposure time, the absorbance at 485 nm was measured by a microwell plate reader (TECAN SpectraFluor Plus, Männedorf, Switzerland).

The cytotoxicity response parameters (GI_{50}) were extrapolated from concentration–response curves.[15]

Genotoxicity on U937 Cells. To verify primary DNA damage, the alkaline version of the Comet assay was carried out on U937 cells, as detailed elsewhere.[16] U937 cells were seeded 24 h before treatment at a concentration of 1×10^5 cells/mL in 1 mL of complete medium. Cells were treated with increasing concentrations (25.0, 50.0, 75.0, and 100.0 μM) of the compounds for 1 and 24 h. DMSO (100 μM) and ethylmethanesulfonate (EMS) (2 mM) were used as negative and positive controls, respectively. After the treatment period at 37 °C, the percentage of live cells was assessed by the Trypan blue exclusion method. Only the treatments with a viability higher than 70% were processed in the Comet assay.[9,16] Percentage of DNA in the tail region of the comet (TI, tail intensity) provided representative data on genotoxic effects. For each sample, coded and evaluated blind, 100 cells were analyzed.

The IBM SPSS Statistics 24 software was used for data analyses, as already described.[9]

Mutagenicity Assessment on Bacteria. To assess the presence of mutagenic activity, the standard plate incorporation method of the Ames test was performed[17] with *Salmonella typhimurium* TA98 and TA100 strains, with and without metabolic activation (S9 mix). Molecules were dissolved in DMSO and assayed at increasing doses (0.1, 1, 10, 50, and 100 μM/plate, corresponding to a range of doses from 0.025 to 25.5 μg/plate and 0.023 to 23.5 μg/plate for molecules **3** and **4**, respectively); negative and positive controls were also introduced.[17] After 48 h, the revertant colonies grown on the plates were counted, and the means of three replicates were computed with their relative standard deviations (net revertants). The results were expressed as the mutagenicity ratio (RM) or the revertants per plate divided by the spontaneous mutation rate (number of revertants in negative controls). They were considered positive if two consecutive dose levels or the highest nontoxic dose level produced a response at least twice that of the control and if at least two of these consecutive doses showed a dose–response relationship.[18,19]

Mutagenicity Assessment on Plants. *Allium cepa Toxicity Test.* Twelve equal-sized young onion bulbs were exposed for 96 h in the dark to different concentrations of molecules dissolved in DMSO, with the sample solution changed every day. Root length was used to calculate the EC_{50} value of each compound and to identify the concentrations to use in the *Allium cepa* genotoxicity assay, in which the highest dose corresponded to the identified EC_{50} value (i.e., the concentration that gives a 50% reduction in root growth). Root macroscopic parameters (turgescence, consistency, change in color, and root tip shape) were used as toxicity indexes.[20]

Allium cepa Genotoxicity Tests. To detect chromosome aberrations and micronuclei,[21,22] six equal-sized young bulbs per sample were exposed to sample solutions for 24 h; then the roots were fixed in acetic acid and ethanol (1:3) for 24 h and finally stored in 70% ethanol for the chromosome aberrations (CA) test.[22] In the micronuclei (MN) test, the bulbs, after exposure, were replaced in saline solution (Rank's solution) for 44 h of recovery time, which covered two rounds of mitosis so that the damage induced in the chromosomes during mitosis would be visible as micronuclei in interphase cells. Then the roots were fixed in acetic acid and ethanol (1:3) for 24 h and finally stored in 70% ethanol.[21] The negative control was DMSO in Rank solution (the dose of DMSO corresponded to the volumes of the samples), and the positive control was maleic hydrazide (10 mg/L, 6 h of exposure). Five roots from each sample were used for microscopic analysis: 1000 cells per slide (5000 cells per sample) were scored for mitotic index (a measure of cell division and hence of sample toxicity), 200 cells in mitosis were scored per slide (1000 cells per sample) for chromosomal aberrations, and 2000 cells in interphase were scored per slide (10 000 cells per sample) for micronucleus frequency.

Statistical analysis was performed using χ^2 tests for mitotic index and chromosomal aberrations, and analysis of variance and Dunnett's *t* tests were performed for MN. All the experiments were in duplicate (two independent assays).

RESULTS AND DISCUSSION

Chemistry. Compounds **1**–**4** (Figure 1) were synthesized by condensation between a ketone and thiosemicarbazide according to a modified literature procedure,[9] and they were

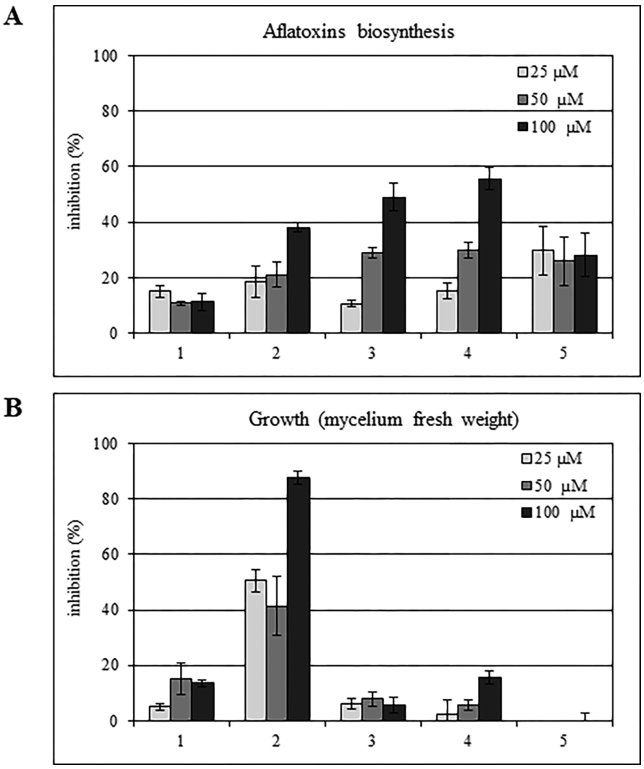

Figure 2. Antifungal and antiaflatoxigenic activities of compounds **1**–**5** at 25, 50, and 100 μM concentrations: (A) aflatoxin accumulation and (B) mycelium fresh weight in CCM medium. The results are expressed as mean percentage inhibition in comparison with the controls (0.25, 0.5, and 1% DMSO, respectively). Error bars indicate the standard deviations of four replicates ($p < 0.05$).

DOI: 10.1021/acs.jafc.9b01814
J. Agric. Food Chem. 2019, 67, 10947–10953

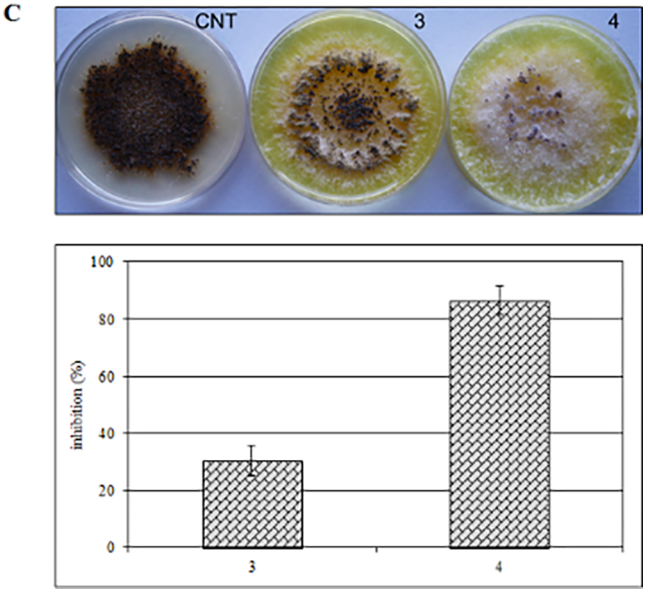

Figure 3. Effects of **3** and **4** on sclerotia biogenesis. Assessments were performed in Czapek Dox Agar solid medium amended with 100 μM thiosemicarbazones or 1% DMSO as the control (CNT). Error bars indicate the standard deviations of three replicates ($p < 0.05$).

Table 1. GI_{50} Values[a] Obtained after 24 h Treatment in Healthy Human Cell Lines CRL1790,[b] Hs27,[c] and HFL1[d] and in Cancer Cell Line U937[e]

	CRL1790	Hs27	HFL1	U937
3	>100.0	>100.0	>100.0	73.0
4	>100.0	79.5	>100.0	58.0

[a]Concentration (μM) of compound that causes a 50% reduction of cell proliferation. [b]Colon epithelial cells. [c]Skin fibroblast cells. [d]Lung epithelial cells. [e]Histiocytic lymphoma cancer cells.

characterized by spectroscopic tools and mass spectrometry (Supporting Information). In their IR spectra, it is possible to observe the NH and NH_2 stretching vibration bands (3430−3140 cm^{-1}), and ν(C═N) at about 1580 cm^{-1}, whereas the C═S stretching absorptions are at about 1035−1080 and 830−950 cm^{-1}. In the ^{1}H NMR spectra recorded in DMSO-d_6, the resonances of the hydrazone NH and of the NH_2 groups (10.3−8.6 and 7.4−8.4 ppm, approximately) are clearly visible. Even if these molecules could give rise to thione−thiol equilibrium, there is not evidence of the presence of the thiolic form in solution.

In our previous investigations,[7−9] we considered the possibility that copper coordination could improve the biodisponibility of the metal ion, favoring adsorption into lipid membranes and thus promoting better antiaflatoxigenic activity. It is well-known that copper salts have been widely applied in agriculture against plant pathogens. In order to further explore this aspect, we synthesized copper complex **5** by reacting **1** with $CuCl_2{\cdot}2H_2O$ in methanol. As we have already observed,[9] in this case the metal center also undergoes reduction to Cu(I). In **5**, the metal is present as a diamagnetic d^{10} Cu(I) ion, and it is in fact possible to record its ^{1}H NMR spectrum in DMSO-d_6 (Supporting Information). It is known from the literature that thiosemicarbazones are subjected to intramolecular oxidative cyclization when some bases,[23] oxidants,[24] or redox-active metals[25] are present. However, the IR and ^{1}H NMR spectra of **5** do not register the presence

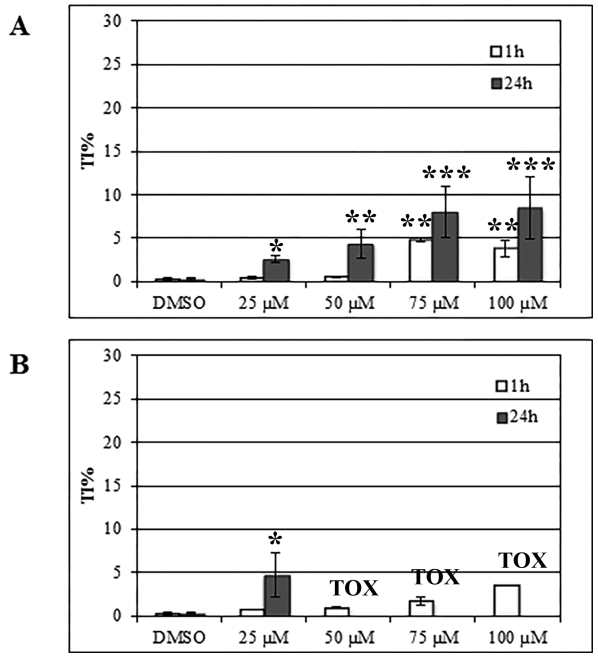

Figure 4. Genotoxic activity of (A) compound **3** and (B) compound **4**, determined by the Alkaline Comet Assay in U937 cells treated for 1 and 24 h. The significance of the difference between the control (DMSO) and the exposure group is indicated by *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (ANOVA, Bonferroni's post hoc test). TOX: viability of <70%.

of cyclized ligand. In the IR spectrum of **5**, the strong bands attributable to the symmetric and asymmetric stretching modes of NH and NH_2 are clearly visible between 3500 and 3000 cm^{-1}. The C═N stretching band shifts from 1582 cm^{-1} (the parent ligand) to 1604 cm^{-1} in the complex, suggesting the coordination of the metal ion to the iminic nitrogen. Also, the stretching band relative to the C═S group is shifted upon coordination from 1073 to 1034 cm^{-1}. In the literature, it is reported that by reacting copper(II) chloride with (+)-camphor and (−)-carvone thiosemicarbazones,[26] polynuclear copper(I) complexes are obtained; in **5**, data analysis suggests a 2:3 metal to ligand stoichiometry. In conclusion, if the monoprotic ligand, **1**, is indicated as HL, for complex **5** it is possible to propose the formula $Cu_2(HL)(L)_2$ (Supporting Information). The $Cu_2(HL)(L)_2$ stoichiometry is also supported by the ESI-MS mass (Supporting Information) and ICP analysis.

Fungal Growth, Aflatoxin Inhibition, and Sclerotia Development. Figures 2 and 3 show the data concerning the effects of treatment with **1**−**5** on AF biosynthesis, biomass accumulation, and sclerotia development.

Compound **1** inhibited AF accumulation only slightly, whereas **2**, **3**, and **4** showed increasing AF inhibition, ranging from 40 to 55% at 100 μM (Figure 2A). Compound **5**, the copper complex of **1**, inhibited AF accumulation slightly more than the ligand alone, but it did not show dose-dependent inhibition, and its antimycotoxin effect was rather scarce. The biomass production evaluation revealed that **2** induced a notable reduction of fresh weight (over 80% inhibition at the highest thiosemicarbazone concentration) (Figure 2B). Poor antifungal activity in conjunction with inhibition of aflatoxin production is highly desirable, because this can realize the preservation of the environmental microbiota while ensuring

DOI: 10.1021/acs.jafc.9b01814
J. Agric. Food Chem. 2019, 67, 10947−10953

Table 2. Results of the Ames Test Expressed as Revertants per Plate and as Mutagenicity Ratios (MRs)

		TA98		TA98 + S9		TA100		TA100 + S9	
sample	dose (μM/plate)	mean ± SD	MR	mean ± SD	MR	mean ± SD	MR	mean ± SD	MR
3	0.1	14.5 ± 0.7	1.0	22.3 ± 4.11	1.0	94.5 ± 19.1	1.2	108.0 ± 0.0	1.2
	1	18.5 ± 2.1	1.2	30.0 ± 1.41	1.3	92 ± 7.1	1.1	99.5 ± 10.6	1.1
	10	11.5 ± 0.7	0.8	20.5 ± 0.71	0.9	96.5 ± 20.5	1.2	83.5 ± 6.4	0.9
	50	19.0 ± 2.8	1.2	22.0 ± 0.0	0.9	100.5 ± 7.8	1.2	86.5 ± 14.8	0.9
	100	16.5 ± 0.7	1.1	20.5 ± 6.36	0.9	95.5 ± 7.8	1.2	79.0 ± 17.0	0.9
4	0.1	12.0 ± 4.2	0.8	15.5 ± 6.36	0.7	75.5 ± 12.0	0.9	86.0 ± 17.0	0.9
	1	17.5 ± 12.0	1.1	26.0 ± 0.71	1.1	77.5 ± 12.0	1.0	86.5 ± 3.5	0.9
	10	15.5 ± 4.9	1.0	16.0 ± 2.12	0.7	74 ± 5.7	0.9	101.5 ± 16.3	1.1
	50	14.5 ± 0.7	1.0	22.0 ± 6.36	0.9	82 ± 1.4	1.0	92.5 ± 10.7	1.0
	100	17.5 ± 3.5	1.1	22.0 ± 3.54	0.9	75 ± 9.9	0.9	75.5 ± 3.5	0.8
negative control[a]		15.25 ± 4.11		23.3 ± 10.6		81.5 ± 4.65		91.3 ± 11.32	
positive controls[b]		>1000		>1000		>1000		>1000	

[a]DMSO (100 μM/plate). [b]TA98: 10 μg/plate 2-nitrofluorene, TA100: 10 μg/plate sodium azide, TA98 + S9: 20 μg/plate 2-aminofluorene, TA100 + S9: 20 μg/plate 2-aminofluorene.

Table 3. *Allium cepa* Chromosomal Aberrations Test on Compound 3: Mitotic Index and Total Chromosomal Aberrations in Metaphase Root Cells

	first experiment			second experiment		
dose (μM)	mitotic index (%)	chromosomal aberration (%)	p^a	mitotic index (%)	chromosomal aberration (%)	p^a
10	12.6	3.5	<0.01	13.3	3.7	<0.01
25	11.6	4.5	<0.01	11.2	4.6	<0.01
50	10.3	4.7	<0.01	10.0	5.1	<0.01
100	12.3	7.4	<0.01	11.8	7.4	<0.01
C−[b]	10.5	1.2		9.9	1.9	
C+[b]	7.2	8.8		8.7	10.1	

[a]Statistically significant according to χ^2 tests. [b]C−, negative control (100 μM DMSO); C+, positive control (10^{-2} M maleic hydrazide).

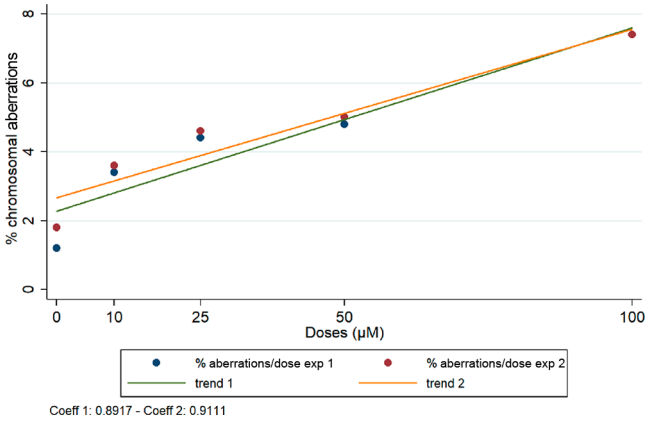

Figure 5. Linear trends of chromosomal aberrations in *Allium cepa* root cells tested with compound **3**.

Table 4. *Allium cepa* Micronuclei Test on Compound 3: Mitotic Index and Total Chromosomal Aberrations in Metaphase Root Cells

	first experiment		second experiment	
dose (μM)	mitotic index (%)	MCN[a] (mean ± SD)	mitotic index (%)	MCN[a] (mean ± SD)
10	11.5	0.03 ± 0.03	11.8	0.03 ± 0.07
25	11.4	0.03 ± 0.03	11.5	0.04 ± 0.04
50	9.6	0.08 ± 0.03	10.4	0.07 ± 0.07
C−[b]	12.5	0.04 ± 0.07	12.6	0.02 ± 0.04
C+[b]	7.6	16.8 ± 8.4	7.2	17.2 ± 12.0

[a]MCN, micronucleus frequency. [b]C−, negative control (DMSO); C+, positive control (10^{-2} M maleic hydrazide).

protection from noxious secondary metabolites. Therefore, **3** and **4** were chosen for further analyses because of their ability to halve AF accumulation without significantly affecting biomass production.

Because AF biosynthesis is known to share several regulatory steps with other developmental processes belonging to the secondary metabolism, such as sclerotia biogenesis,[27] the effect on the production of these structures was assessed. Sclerotia formation was induced by culturing the aflatoxigenic and sclerotigenic *A. flavus* strain CR10 in CZA solid medium amended with 100 μM of **3** and **4** and 1% DMSO as control. The presence of the thiosemicarbazones in the culture medium reduced the formation of sclerotia, whereas no mycelium growth inhibition was observed, as expected. With respect to the control, exposure to **3** and **4** limited sclerotia biogenesis to 30 and 86%, respectively (Figure 3). These results are in line with our previous observations regarding other thiosemicarbazones that are effective in inhibiting AF production but have a slight effect on fungal growth and severely impair sclerotia development in *A. flavus*.[7]

Cytotoxicity. The cytotoxicities of the most interesting compounds, **3** and **4**, were screened over a panel of human cell lines. Three normal healthy cell lines were chosen to represent possible different routes of exposure to chemicals: epidermal contact (skin fibroblast, Hs27), inhalation (lung epithelial cells, HFL1), and ingestion (colon epithelial cells, CRL1790). On the other hand, tumor cell line U937 is a model commonly used to identify cytotoxicity and genotoxic activity of drugs.[28]

DOI: 10.1021/acs.jafc.9b01814
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Growth inhibition (GI) was determined by an MTS assay; data are means of four independent experiments (Table 1).

Compound **3** showed no antiproliferative activity against colon, skin, and lung cell lines and presented a mild cytotoxic effect only on cancer cells (Table 1). A mild cytotoxic effect against skin fibroblast cells and more pronounced cytotoxicity on U937 cells (Table 1) were observed with **4**.

Because **3** and **4** present good profiles in term of cytotoxicity on normal cell lines, they were considered for further analysis.

Genotoxicological Assessment. The Alkaline Comet Assay was performed on U937 cells to identify the genotoxic potentials of **3** and **4**. The test is able to measure DNA damage and is usually carried out at pH > 13 to detect, in addition to single and double strand breaks, alkali-labile sites such as adducts, apurinic and apyrimidinic sites, and oxidized nitrogenous bases, among others. The percentage of DNA in the tail of the comet (TI%) provides representative data on the genotoxic effects of the molecules. Cells were treated with 25.0, 50.0, 75.0, and 100.0 μM concentrations of the molecules for 1 and 24 h. Against U937 cells, compound **3** induced DNA strand breaks in a dose-dependent manner over both treatment times (1 and 24 h) (Figure 4A). Compound **4** after 1 h of treatment induced DNA damage at the highest concentrations (75.0 and 100.0 μM), whereas after 24 h it was already genotoxic at 25.0 μM (Figure 4B); it caused, as expected, reductions in cell viability and showed toxic effects at the highest concentrations.

The results of the Ames test showed that **3** and **4** had no mutagenic activity in *Salmonella typhimurium* strains TA98 and TA100, with and without metabolic activation (S9) (Table 2).

Unfortunately, in preliminary tests, solutions of **4** exposed to the roots of *Allium cepa* showed high toxicity at even the lowest doses, with decreases in the number of cell division (too low of a mitotic index); for this reason, it was not possible to carry out a genotoxicity test. In contrast, for **3**, the dose corresponding to the EC_{50} was evaluated by a root toxicity test, and it was used as the maximum dose for further genotoxicity evaluations. The molecule had an EC_{50} of 50 μM, with slight toxic activity at the highest tested dose (100 μM). The test of chromosomal aberrations in *Allium cepa* showed clear genotoxic activity for **3** already at the lowest dose tested, with a statistically significant increase in chromosomal aberrations compared with the negative control, without a reduction in the number of cell divisions or mitotic index (Table 3).

In the linear trend analysis, the sample gave a positive response, showing a clear dose−response relationship that was statistically significant ($p < 0.05$) (Figure 5).

The frequency of micronuclei in interphase cells of *Allium cepa* is reported in Table 4: there is no increase of micronuclei compared with the negative control for compound **3**.

In conclusion, we were focused on finding specific inhibitors of AF biosynthesis rather than inhibitors of fungal growth, and benzophenone thiosemicarbazone **3** and valerophenone thiosemicarbazone **4** seem interesting in this sense. Compounds **3** and **4** are promising also because they show no antiproliferative activity against colon, skin, and lung cell lines and are characterized by the absence of mutagenic activity on bacteria. However, the Alkaline Comet Assay performed on U937 cells points out that **3** and **4** produce DNA damage. Compound **4** is toxic to the roots of *Allium cepa*, and a test of chromosomal aberrations in *Allium cepa* showed clear genotoxic activity for **3**, even at the lowest doses. The *Allium cepa* micronuclei test was negative, however: compound **3** caused directly DNA damage and chromosomal alterations but did not cause disturbances in the mitotic cycle or damage to the mitotic spindle.

Compounds **3** and **4** are promising hit compounds, and the next efforts will be devoted to investigating the action mechanisms of the antiaflatoxigenic thiosemicarbazones and controlling their genotoxic character.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b01814.

IR and ^{1}H NMR spectra of compounds **1**, **2**, **3**, and **4** and IR, ^{1}H NMR, and mass spectra of **5** (PDF)

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§J.B., S.M., and G.S. are considered cofirst authors. The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript. D.R., J.B., M.C., F.B, and G.P. were involved in molecular design and chemical synthesis; F.M.R., F.D., and G.S. were involved in fungal growth, sclerotia development, and aflatoxin inhibition evaluation. A.B., S.M., and O.S. were involved in the cyto- and genotoxicological assessments on human cells. D.F. and C.Z. were involved in the genotoxicological assessments on bacteria and plants.

Funding

This study was supported by a grant from "Fondazione Cariplo" (Project No. 2014-0555, http://aflatox.it).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank "Centro Interdipartimentale Misure Giuseppe Casnati" of the University of Parma for use of facilities.

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