Design and Synthesis of Metal Complexes as Antifungals to Protect Environment and Human Health against Aflatoxins

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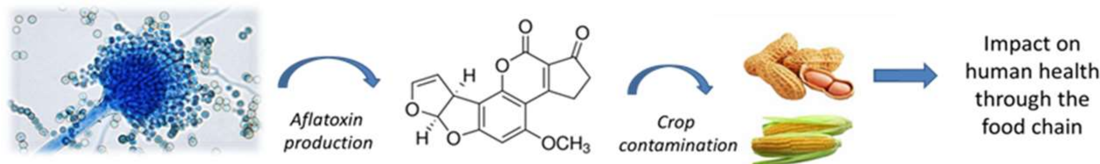
Background

Aflatoxin contamination has a strong impact on the quality and safety of food and feed products worldwide because of their genotoxic and carcinogenic potential [1]. Aflatoxins are a group of furanocoumarin derivatives mainly produced by toxigenic strains of *Aspergillus flavus* and *parasiticus*: they are highly thermostable in nature and are not degraded even during cooking process. The direct control of mycotoxin-producing fungi by using synthetic fungicides is still the most effective way to intervene, but concerns on food safety and environmental health, combined with the global issue of emerging resistant pest strains, make urgent to **develop novel crop-protective agents**.

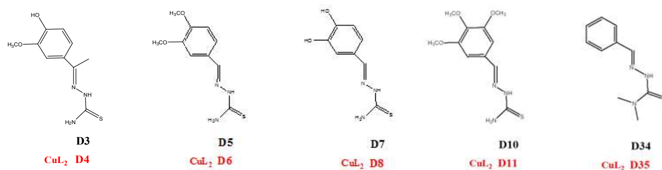
Many natural products and their chemical analogues have been successfully developed as crop-protective agents [2]. On the other hand inorganic substances, like copper salts, have been long used for their capacity of inhibiting the development of moulds and bacteria. The lipidic membrane that surrounds the cell constitutes a barrier to metal ions diffusion, but small hydrophobic molecules can easily diffuse through this barrier, and for this reason lipophilicity is an important factor controlling the antifungal activity. **Metal chelation** could improve lipophilicity facilitating the penetration of the complexes into lipid membranes, and should restrict proliferation of the microorganisms.



Results

Synthesis

A series of thiosemicarbazonic ligands derived from natural aldehydes were synthetized. Reaction between the ligand and $CuCl_2$ in methanol leaded to the precipitation of the metal complex. Analysis of data (IR, MS-ESI, elemental analysis, UV) indicate the presence of copper(II) coordination compounds, where the ligand coordinates to the metal centre through the iminic nitrogen and the sulfur atom in a 1:2 metal to ligand ratio.



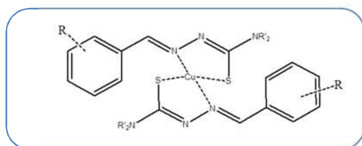
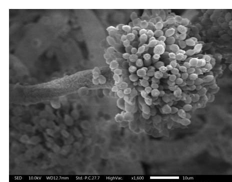


Figure 1

Antifungal and antimycotoxin activity

Both ligands and copper complexes were tested for their ability to inhibit growth inhibition of *Aspergillus flavus* and aflatoxin accumulation at different concentration (**Table 1**). Generally metal complexes inhibit aflatoxin production better than the corresponding ligand (see **D4**, **D6** and **D11** compared to **D3**, **D5**, **D10,** respectively). Moreover, activity on aflatoxin production of these copper complexes is jointed with good fungal growth inhibition. An exception is represented by **D8**; also the corresponding ligand **D7** is devoid of activity.

D34 and its copper complex **D35** show the best activity.



	Growth inhibition (%)		Inhibition of aflatoxin production (%)	
	50 µM	100 µM	50 µM	100 µM
D3	52	50	26	12
D4	39	28	52	72
D5	80	97	26	25
D6	59	49	62	80
D7	100	100	5	21
D8	-	-	0	6
D10	100	100	6	0
D11	45	46	47	76
D34	78	56	76	90
D35	100	100	35	67

Table 1. Antifungal and inhibition of aflatoxin production activities expressed respectively as mean percentage of growth inhibition and as mean percentage of inhibition aflatoxin production (compared with non-treated controls)

Cytotoxicity on human cells

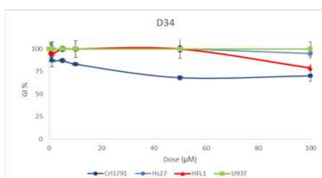
A screening of the toxicity of the most active compounds in term of aflatoxin inhibition (**D4, D6, D11, D34, D35**) was performed over a panel of human normal cell lines.

Growth inhibition (GI) determination was performed by MTS assay. Unfortunately, all the copper complexes showed important cytotoxicity in the micromolar range

The ligand **D35** has very low effect on cell proliferation. Therefore, it was chosen for further genotoxicity analysis on bacteria and plants.

	Crl1791	Hs27	HFL1	U937
D34 (µM)				
0	100±0	100±0	100±0	100±0
0,5	94,6±3,9	100±5,3	96,7±2,1	100±6,7
1	87,2±6,8	100±3,8	94,52±3,7	100±7,8
5	87,2±2,5	100±2,9	100±2,4	100±2,3
10	83,2±1,5	100±1,3	100±2,4	100±9,2
50	68,2±2,3	100±2,9	100±3,4	100±10,3
100	70,3±6,3	95,0±4,7	78,9±3,4	100±8,0
D35(µM)				
0	100±0	100±0	100±0	100±0
0,5	91,3±	100±4,0	100±0	100±0
1	59,9±5,3	93,1±3,0	47,1±13,8	95,6±2,8
5	21,5±1,5	13,6±6,0	14,6±9,4	38,3±2,0
10	20,4±1,5	14,7±0,4	17,3±1,3	40,5±1,3
50	21,9±2,3	17,6±0,6	28,8±1,5	64,5±1,7
100	28,6±1,2	22,4±0,4	33,4±7,2	66,9±6,2

Table 2. Growth inhibition (%) on different cell lines treated with **D34** and **D35** at different concentrations.



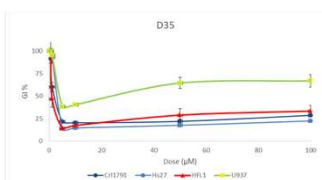


Figure 2. Dose-response curves for **D34** and **D35** in different cell lines

Genotoxicity on bacteria and plants

D34, the most active ligand with negligible citotoxicity towards human cells, was tested using short-term mutagenicity tests with different genetic end-points on bacteria and plants. Ames test was conducted with *S. typhimurium* strains TA100 and TA98, with and without microsomal activation (S9 fraction). The results were expressed as Mutagenicity Ratio (MR). Results were considered positive if two consecutive dose levels were at least twice than negative control (**Table 3**). Moreover, toxicity tests were made on *Allium cepa*: in preliminary assays, equal-sized young bulbs of onions were exposed for 72 hours in the dark to different concentrations of the thiosemicarbazone. The length of the roots was used to calculate the EC_{50} value of the compounds and to identify the concentrations to test in *A. cepa* genotoxicity assay. *A.cepa* pre-germinated bulbs were exposed to the tested compound for 24 hours. The slides were observed at 1.000x: 5.000 cells per sample were scored to determine mitotic index (MI) and 10,000 cells per sample to determine frequency of micronuclei (MCN) (**Table 4**).

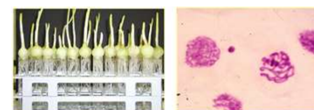
D34 exhibited no mutagenicity in the bacterial test on *S. typhimurium* TA100 and TA98 strains with or without metabolic activation at all tested doses. The *A. cepa* test showed toxicity on roots at 100 µM, but for lower doses no genotoxic effects were observed.

DOSE (µM/plate)	TA 98		TA98+S9		TA 100		TA100+S9	
	number of revertants colonies per plates (± Standard Deviation)	MR	number of revertants colonies per plates (± Standard Deviation)	MR	number of revertants colonies per plates (± Standard Deviation)	MR	number of revertants colonies per plates (± Standard Deviation)	MR
Negative control	19,0 ± 6,4		36,2 ± 6,0		109,5 ± 9,9		127,3 ± 8,1	
0,1	10,0 ± 2,8	**0,5**	34,0 ± 1,4	**0,9**	128,0 ± 14,1	**1,2**	143,0 ± 7,1	**1,1**
1	15,5 ± 7,8	**0,8**	33,5 ± 4,9	**0,9**	116,0 ± 11,3	**1,1**	132,0 ± 9,9	**1,0**
10	17,0 ± 2,8	**0,9**	35,0 ± 4,2	**1,0**	105,5 ± 6,4	**0,9**	132,5 ± 7,8	**1,0**
50	21,0 ± 2,8	**1,1**	36,5 ± 0,7	**1,0**	135,5 ± 20,5	**1,1**	94,5 ± 2,1	**0,7**
100	17,0 ± 4,2	**0,9**	40,0 ± 5,7	**1,1**	105,0 ± 1,4	**1,0**	138,0 ± 5,7	**1,1**

Table 3. Mutagenicity Ratio (MR) obtained from mean number of revertants colonies per plates for negative controls with **D34**

D34	MI (%)	MCN (mean ± SD)	Negative controls
50 µM	7.1	0.07±0.06	0.04±0.07
25 µM	10.2	0.1±0.08	0.01±0.02
10 µM	7.7	0.1±0.2	0.03±0.07

Table 4. Mitotic index (MI) and micronuclei frequency (MCN) in *A. cepa* roots treated with **D34**



References

[1] World Mycotoxin J., 8 (2015) Special Issue: Issue 2

[2] Hüter, O. Phytochem. Rev., 10 (2011) 185

fondazione cariplo

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