



Triazoles and aromatase: The impact of copper cocktails[☆]

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ABSTRACT

Triazoles are used as antifungal agents, they mostly inhibit two enzymes: 14 α -demethylase and aromatase. These enzymes are utilised also in other species and therefore the affection in non-target species in the environment is expected as well. Besides, triazoles are often being applied in a mixture and they can also interact with other substances present. This study clarifies how three selected representative triazoles (tebuconazole, penconazole and cyproconazole) interact with each other (group effect) and in mixtures (cocktail effect) with copper, essential/toxic for all organisms. Within the experiments on electrospray and collision-induced dissociations (both ESI-MS), it has been found that the fragments correspond to typical triazole metabolites. For their formation, the presence of copper ions is crucial. The inhibitory effect of Cu cocktails on aromatase enzymatic activity has been studied. The presence of Cu ions together with triazole(s) significantly increases the inhibitory effect on aromatase activity. The highest inhibitory effect (more than 60%) on aromatase activity is produced by cocktails containing penconazole and Cu ions, namely by penconazole/Cu and penconazole/tebuconazole/Cu. The reactivity of triazoles in groups is not significantly affected by the interactions among them. Additionally, the role of triazoles in copper Fenton reaction regulation has been observed and described. These changes may be attributed to the formation and stabilization of the complexes with the central Cu ion, with usually one, two or three triazolic ligands, depending on the mixture. The study demonstrates that the interaction of triazoles and Cu ions is a complex process; their impact on metabolism seems to be rather extensive and must be evaluated in the context of biochemical reactions.

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1. Introduction

Triazoles (belonging to the group of azoles) are systemic fungicides frequently used to crop protection against yeast and mildew. In these target species, azoles inhibit the biosynthesis of steroids (Egbuta et al., 2014; Zarn et al., 2003) mainly by blocking fungal cytochrome P450 monooxygenase sterol 14 α -demethylase

(CYP51A1, EC 1.14.14.154). The inhibition of fungal CYP51 leads to the depletion of ergosterol, essential in fungal plasma membranes and thus the destabilization of the membrane (Alcazar-Fuoli et al., 2008). For humans, inhibition of CYP51 is not crucial because diet supplies cholesterol (an intermediate).

Many azoles also inhibit aromatase (CYP19A1, EC 1.14.14.14) (Conner et al., 2012). Cyp19 catalyses the conversion of androstenedione, testosterone or 16 α -hydroxytestosterone to estrone, 17 β -estradiol or 17 β ,16 α -estradiol, respectively (Conley et al., 2001; Tröskén et al., 2004). The depletion of estrogens is the principle of the use of azoles in the antiestrogen therapy against estrogen-dependent adenocarcinoma and breast cancer cells (Egbuta et al., 2014; Lang et al., 1993).

Since most azoles are not completely specific, they are able to inhibit additional key cytochrome P450 enzymes, e.g. lanosterol-14-demethylase (CYP1) (Beijer et al., 2018), cholesterol side-chain

Abbreviations: Cyp, cyproconazole; Pen, penconazole; RFU, relative fluorescence units; Teb, tebuconazole; AE, appearance energy.

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cleavage (CYP11A) steroid 11 β -hydroxylase (CYP11B and CYP11C1) and steroid 17 α -hydroxylase/17,20 lyase (CYP17) responsible for transformation of steroid hormone (Chen and Ying, 2015).

These effects on highly conserved cytochrome P450 monooxygenases such as CYP51, are similar to those observed in mammals including human (Chen and Ying, 2015; Lepesheva et al., 2003). In healthy non-target organisms, azoles are consequently considered to be potential endocrine disruptors (Taxvig et al., 2008; Trösken et al., 2004; Zarn et al., 2003).

The individual influence of various azoles on the target enzymes has been well described. Because of the treatment strategies, the issue of the possible effect of (tri)azoles in a mixture (cocktail effect) on given enzymes is of great interest (Kjaerstad et al., 2010; Rieke et al., 2014; Seeger et al., 2019; Schmidt et al., 2016).

Triazoles as fungicides are widely used in agriculture, in the field from which they easily spread through the soil; and contaminate groundwater and even reach urban centres. Moreover, these compounds are also used in therapeutic and personal care products (shampoos, dermal creams, and soaps etc.) (Assres et al., 2020). Their removal rate in wastewater treatment plant is rather low. Azole fungicides are generally detected in surface water and sediment of the aquatic environment (Chen and Ying, 2015). In our previous studies it was found that the behaviour of the triazoles and their degradation pathways are also strongly influenced by their reactions with the ions of various elements present (Jaklová Dyrtrtová et al., 2014; Kovač et al., 2020).

Besides triazoles, especially copper compounds are also widely applied as fungicides for crops (Komárek et al., 2010; Vazquez-Blanco et al., 2020). There has been evidence that triazole fungicides can create complexes with copper ions and change their behaviour in the environment, i.e., their accumulation in soils, degradation, mobility, and stability (Arias et al., 2006; Jakl et al., 2017; Jakl and Jaklová Dyrtrtová, 2018; Jaklová Dyrtrtová et al., 2011). Copper as an ion coordinated in many enzymes plays an essential role in the biochemistry of all living organisms. On the other hand, its free ions in cells may be toxic via the catalysis of Fenton (Haber-Weiss) reactions forming reactive oxygen species and hydroxyl radicals. In addition to the oxidative damages, the presence of free Cu ions may lead to the direct impairment of redox-sensitive signalling and to functional changes of activator protein 1 (AP-1), a transcription factor regulating gene expression (Letelier et al., 2009). In the presence of copper ions, the activity of cytochrome P450 monooxygenases could be passivated by their oxidation, by copper ions binding to their amino acids and by other structural changes (Letelier et al., 2009).

This research has focused on three triazolic compounds: tebuconazole, penconazole and cyproconazole, which are often used in crop protection. Tebuconazole is even the world's most widely used triazolic fungicide (as a seed dressing and foliar spray against rusts, smuts, bunt, powdery mildew, leaf spots, and blights) with the potentially most harming impact on the environment (Li et al., 2019; Sharma et al., 2019). Only a few studies have been focused on penconazole, however its toxic effects were reported on various organisms, including mice and fish (Aksakal and Ciltas, 2018; Meng et al., 2020). Cyproconazole has been widely used in restraining and curing plant diseases and deeply studied since it was shown to have stereoselective toxicity against *Chlorella pyrenoidosa* and *Pelophylax nigromaculatus* (black-spotted frog) (He et al., 2019).

All of these compounds have a relatively similar structure (see the Graphical Abstract), which concerns not only the nitrogen heterocycle but also the chlorinated benzene ring on their aliphatic chains. On the contrary, they do not contain other halogens or epoxy chains, which could fundamentally change their reactivity. The main aim here is to bring the information on how the cocktails of these similar triazoles in their different (typically racemic)

mixtures and with copper ions influence the activity of aromatase and thus to predict their environmental behaviour and impact.

2. Material and methods

2.1. Chemicals

Tebuconazole (**Teb**; 1-(4-chlorophenyl)-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol), penconazole (**Pen**; 1-[2-(2,4-dichlorophenyl)pentyl]-1,2,4-triazole) and cyproconazole (**Cyp**; 2-(4-chlorophenyl)-3-cyclopropyl-1-(1H-1,2,4-triazol-1-yl)butan-2-ol) were of analytical grade purity (Pestanal®), and CuCl₂ of 99.999% (trace metal basis) purity. All of these chemicals were purchased from Sigma-Aldrich (Czech Republic). The solid standards were used for the preparation of stock solutions. The stock solutions of triazoles (10⁻² mol/L) were prepared in acetonitrile (Sigma-Aldrich) for biochemical experiments or in ethanol (99.9%; VWR s.r.o.) and the stock solutions of metal salt (CuCl₂) were prepared in deionised water (18.2 M Ω purity, Milli-Q®, Millipore). All the stock solutions were stored in a freezer (-25 °C). A rigid number of heterocyclic rings in the mixtures was required. The overall problem was focused on equimolar mixtures of triazoles with unchanged total heterocycles concentration. This approximation allows to use average to estimate hypothetical values, which may serve as comparison for the experimental values.

2.2. Effect on human recombinant aromatase (CYP19A) studied in vitro

The effect of the test triazoles and Cu ions on aromatase activity was evaluated in the samples prepared with the equimolar concentration of triazoles (10⁻⁶ mol/L) with/without the presence of CuCl₂ equimolar to their sum (Table 1). The activity of aromatase was experimentally determined using a NADPH-generating system (based on glucose-6-phosphate dehydrogenase) and a fluorogenic substrate of two-fold K_m concentration, which after reduction turns into a highly fluorescent metabolite. For this purpose, the Aromatase (CYP19A) Inhibitor Screening Kit (Fluorometric) (BioVision, Milpitas, USA) was used. The stock solutions of triazoles were prepared in acetonitrile (Sigma-Aldrich) because it was shown to have the least impact on aromatase activity at the final concentration $\leq$ 1% according to the manufacturer. The aromatase substrate was also dissolved in acetonitrile according to the manual (0.12% in reaction). The presence of 10⁻⁶ mol/L triazole solution meant an only 0.01% increase in the total acetonitrile concentration in the reaction mixture. The increase of acetonitrile had no effect on aromatase activity (data not shown). The fluorescent metabolite was measured using a multi-well fluorescence microplate reader (Infinite M200 PRO, Tecan, Switzerland) in the kinetic mode at 488/527 nm (Ex/Em) according to the manufacturer's instructions (60 min, 37 °C) with the use of white 96-well plates with a flat bottom (Costar, Corning, USA).

The activity of aromatase was expressed in percentage, whereas the activity of 100% was related to the reference control sample containing no triazoles. The activities measured in single-component samples (consisting of 10⁻⁶ mol/L **Teb** or **Pen** or **Cyp** or CuCl₂) served for the estimation of the hypothetical activity (A_{hypot}) of aromatase in multicomponent samples. For instance, in the sample that consisted of an equimolar mixture of **Teb**/**Pen**/**Cyp** (3.33·10⁻⁷ mol/L each, triazoles together 10⁻⁶ mol/L), the hypothetical activity was calculated as the average of the experimental activities (A_{exp}) determine in the single-compound samples:

$$A_{\text{hypot}} = \frac{A_{\text{exp}}(\text{Teb}) + A_{\text{exp}}(\text{Pen}) + A_{\text{exp}}(\text{Cyp})}{3}$$

Table 1

Activity of aromatase in different reaction mixtures. The activity of the control sample was 1.3 ± 0.1 RFU/min/ μ g. The different letters above each reaction mixture denote significant differences ($P \leq 0.05$) between the groups according to one-way ANOVA. The same minuscule letters indicate no statistically significant differences. A_{exp} ... experimental activity, A_{hypot} ... hypothetical (calculated) activity, D ... hypothetical decrease of aromatase activity. The significant difference between A_{exp} and A_{hypot} indicated by * was analysed by *t*-test ($P \leq 0.05$).

Reaction mixture	A_{exp} (%)	error	A_{hypot} (%)	error	$A_{\text{exp}}-A_{\text{hypot}}$	aromatase activity
Control (buffer) ^{a,f}	100.00	2.60				
Teb ^{b,g}	77.30	10.10				
Pen ^c	41.00	5.30				
Cyp ^{b,d}	72.30	8.00				
Teb-Pen-Cyp ^{b,d,e}	69.20	8.40	63.53	6.00	5.67	
Teb-Pen ^{c,d}	55.30	9.80	59.15	7.70	-3.85	
Teb-Cyp ^{a,b}	81.20	5.00	74.80	9.05	6.40	
Pen-Cyp ^{c,e}	50.10	8.00	56.65	6.65	-6.55	
Cu ²⁺ , f	118.50	2.90				
Teb-Cu ²⁺ , b,d	72.10	3.20	97.90*	10.10	-25.80	D
Pen-Cu ^{2+,c}	39.10	2.60	79.75*	5.30	-40.65	D
Cyp-Cu ^{2+,b,d,e}	70.10	3.90	95.40*	8.00	-25.30	D
Teb-Pen-Cyp-Cu ^{2+,c,d,g}	58.80	9.50	93.85*	6.00	-35.05	D
Teb-Pen-Cu ^{2+,c}	48.20	1.50	88.83*	7.70	-40.63	D
Teb-Cyp-Cu ^{2+,a,b}	79.80	5.50	96.65	9.05	-16.85	
Pen-Cyp-Cu ^{2+,c}	47.80	6.40	87.58*	6.65	-39.78	D

The A_{hypot} for the mixture containing an equimolar mixture of **Teb**/**Pen**/**Cyp** ($3.33 \cdot 10^{-7}$ mol/L each, triazoles together 10^{-6} mol/L) and 10^{-6} mol/L of CuCl_2 is expressed as:

$$A_{\text{hypot}} = \left(\frac{A_{\text{exp}}(\text{Teb}) + A_{\text{exp}}(\text{Pen}) + A_{\text{exp}}(\text{Cyp})}{3} + A_{\text{exp}}(\text{Cu}) \right) / 2$$

The determinations were performed in three replicates, each together with a reference control sample (without azoles and metals). The data were analysed by One Way ANOVA (Tukey method, Sigmaplot 12.5, Systat Software, Inc.); the differences were considered statistically significant at $P \leq 0.05$.

2.3. Creation of metal/triazole adducts studied using ESI-MS

The experiments were performed using a quadrupole ion-trap mass spectrometer (Finnigan LCQ Advantage, ThermoFinnigan, USA) in the positive mode. The samples were prepared from the stock solutions of triazoles and metals (10^{-2} mol/L) in a manner similar to that used in the preparation of the samples from biochemical experiments (to keep the same proportion of mixing – see Table 1). In contrast, the total concentration of azoles (as well as metals) in the samples was 50 times higher because of the sensitivity of the instrument. For example, the sample **Teb-Pen-Cyp-Cu**²⁺ (in Table 1) contained $5 \cdot 10^{-5}$ mol/L of CuCl_2 and $1.67 \cdot 10^{-5}$ mol/L of each triazole (**Teb**, **Pen** and **Cyp**). The sample solutions were continuously fed into the ion source at 0.5 mL/h through a silica capillary. Nitrogen was used as the nebuliser gas and sweep/auxiliary gas (8–15 arbit. units) and helium as the collision gas. The other conditions of the ion source were set as follows: spray voltage: 4.50 kV, capillary temperature: 200 °C, capillary voltage 20 V, tube lens offset 15 V.

Collision-induced dissociation (CID) experiments were used for the study of the dissociation of metal-triazole complexes. The methodology was based on the dissociation of benzylpyridinium ions (Zins et al., 2010). The mass spectra of mass-selected ions were measured as fragmentation thresholds in percentages of normalised collision energy (%NCE), optimised by the manufacturer in the range of 0–100%, and corresponded to a resonance-excitation alternating-current signal of 0–2.5 V (zero-to-peak) (Zins et al., 2010). Normalised collision energies were converted into kilojoules per mole via phenomenological conversion; phenomenological appearance energies (AEs) are in correlation with the NCE

framework (Révész et al., 2010). The phenomenological conversion factor (which is an individual parameter depending on the instrument) was experimentally determined as follows: E_{coll} (kJ/mol) = $(7.3 \pm 0.3) \cdot E_{\text{coll}} (\%)$.

3. Results and discussion

Each individually assessed triazole significantly reduced the aromatase activity (Table 1), in the case of penconazole even to 41%. Their combinations had a similar effect as was expected from hypothetical calculations. On the other hand, the addition of Cu^{2+} to the triazole mixtures decreased the aromatase activity in contrast to the hypothetical expectations. It is generally assumed that the effect of all triazoles is more or less similar. However, it is visible from our results (Table 1) that this assumption is completely wrong.

In the first three lines of Table 1, showing the activities of aromatase in the presence of **Teb**, **Pen** and **Cyp** (as singles), it is visible that the biggest inhibitory effect on aromatase is produced by **Pen**, followed by **Cyp** and **Teb**.

To understand the possible **group effect** (which can be attributed e.g. to some inter-reactions) of these three triazoles, we prepared their mixtures. The hypothetical activity of aromatase is estimated as a simple addition. The difference from this hypothetical (additive) activity can be ascribed to the group effect. It arises from the lines 4–7 of Table 1, that this group effect is small (on the level of error) and not statistically significant. This result means that there are no significant reactions or interactions among these three triazoles. This fact can also be supported by the absence of those products in the mass spectra (Fig. S11–3).

If we switch to a potentially more reactive system – triazoles with copper cations, we can estimate the **cocktail effect** of those triazole/copper mixtures from the hypothetical activity (assumed as a simple addition) and the experimental activity. From the last part of Table 1, it is visible that the difference between the experimental and hypothetical activity is bigger than the error and therefore statistically significant ($P \leq 0.05$). Additionally, the cocktail effect of copper/triazole mixtures reduces the aromatase activity in contrast to hypothetical expectation.

The biggest cocktail effect, decreasing the aromatase activity by more than 40% (in comparison with hypothetical expectations), has been observed for the mixtures of **Pen** and Cu^{2+} , **Teb/Pen** and Cu^{2+} , and **Cyp/Pen** and Cu^{2+} . All of these mixtures contain **Pen**. **Pen** in the presence of Cu^{2+} is transformed into several metabolites (Kováč

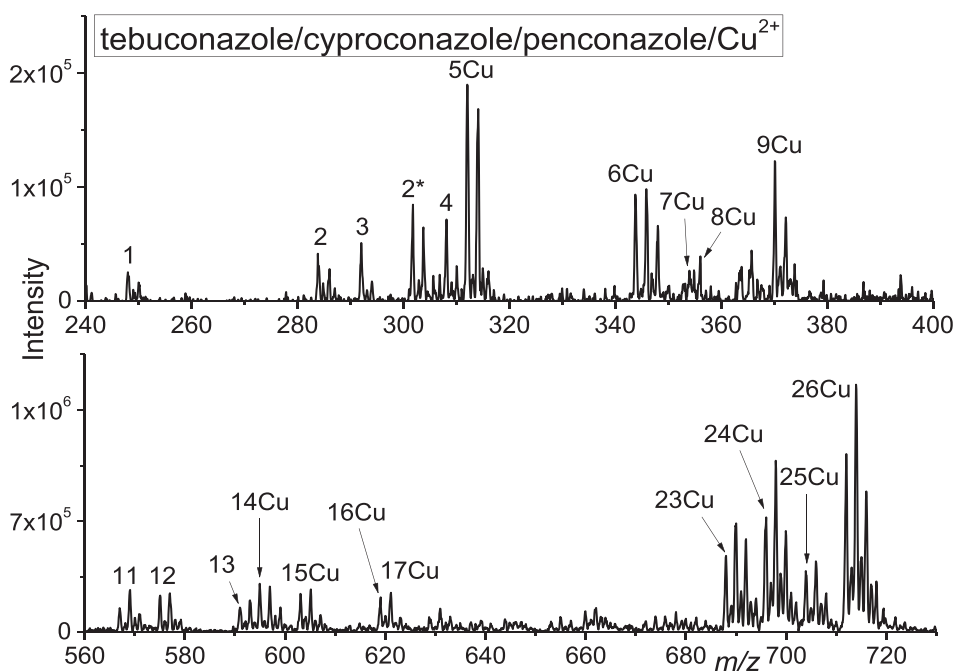


Fig. 1. Full MS spectrum of the mixture of CuCl_2 ($5 \cdot 10^{-5}$ mol/L), tebuconazole ($1.67 \cdot 10^{-5}$ mol/L), cyproconazole ($1.67 \cdot 10^{-5}$ mol/L) and penconazole ($1.67 \cdot 10^{-5}$ mol/L) in the ESI positive mode. The cations are listed in Table 2.

et al., 2020). These adducts are potentially more effective in aromatase inhibition than single penconazole.

It is evident that the interactions of triazoles with copper ions influence their effect on aromatase. It is essential to know which reaction products are formed. For this purpose, it is useful to use the gas phase approach to study the variety of possible reactions or complexes using ESI-MS. The data obtained must, of course, be thoroughly evaluated, because the experiments in the gas phase do

not always give the same picture that is provided in the liquid phase (Agrawal and Schröder, 2011; Schröder, 2004).

According to our previous results (Jakl et al., 2017; Jaklová Dyrtrtová et al., 2014), the reactivity of triazoles in the presence of some metals might be modified via the creation of complexes or at least of metabolites. Our previous studies (Jakl et al., 2017; Jaklová Dyrtrtová et al., 2014; Jaklová Dyrtrtová et al., 2018; Norková et al., 2012) have also shown that triazoles (**Cyp** and **Teb**) bind to

Table 2

List of all the cations present in the full spectra (Fig. 1) of CuCl_2 ($5 \cdot 10^{-5}$ mol/L), tebuconazole (**Teb**, $1.67 \cdot 10^{-5}$ mol/L), cyproconazole (**Cyp**, $1.67 \cdot 10^{-5}$ mol/L) and penconazole (**Pen**, $1.67 \cdot 10^{-5}$ mol/L).

no.	m/z	formula	absolute intensity	relative intensity (%)	AE (kJ/mol)
1	248.10	(Pen-Cl) ⁺	3.64E+04	0.74	216
2	284.07	(HPen) ⁺	3.21E+04	0.66	190
3	292.12	(HCyp) ⁺	8.20E+04	1.68	189
2*	302.08	(HPen) ⁺ (H ₂ O)	8.40E+04	1.72	156
4	308.15	(HTeb) ⁺	7.09E+04	1.45	174
5Cu	312.00	[Cu(Teb*)]⁺/[Cu(Cyp*)]⁺/[Cu(HPen-Cl)]⁺	2.11E+05	4.31	219
6Cu	345.99	[Cu(Pen)] ⁺	8.83E+04	1.80	164
7Cu	353.04	[Cu(Cyp-H)] ⁺	1.86E+04	0.38	12
8Cu	354.04	[Cu(Cyp)] ⁺	2.86E+04	0.58	168
9Cu	370.08	[Cu(Teb)] ⁺	8.52E+04	1.74	224
10Cu	405.04	[Cu(Teb)Cl] ⁺	2.81E+04	0.57	138
11	567.14	[H(Pen)₂]⁺	1.65E+05	3.37	208
12	575.19	[H(Pen)(Cyp)]⁺	2.23E+05	4.56	183
13	591.22	[H(Teb)(Pen)]⁺	1.71E+05	3.49	157
14Cu	595.10	[Cu(HPen-Cl)(Pen-H)] ⁺	2.07E+05	4.23	188
15Cu	603.15	[Cu(HPen-Cl)(Cyp-H)] ⁺	2.47E+05	5.05	175
16Cu	629.06	[Cu(Pen)₂]⁺	1.07E+05	2.19	197
17Cu	637.11	[Cu(Pen)(Cyp)] ⁺	6.84E+04	1.40	178
18Cu	644.16	[Cu(Cyp)(Cyp-H)] ⁺	8.40E+04	1.72	12
19Cu	645.16	[Cu(Cyp) ₂] ⁺	8.40E+04	1.72	188
20Cu	653.16	[Cu(Teb)(Pen)] ⁺	4.87E+04	1.00	165
21Cu	660.19	[Cu(Teb)(Cyp-H)]⁺	1.35E+05	2.76	201
22Cu	661.19	[Cu(Teb)(Cyp)] ⁺	8.65E+04	1.77	158
23Cu	688.11	[Cu(Teb)(Pen)Cl]⁺	4.16E+05	8.50	187
24Cu	696.16	[Cu(Teb)(Cyp)Cl]⁺	7.27E+05	14.86	195
25Cu	704.00	[Cu(Pen) ₂ (C ₃ H ₅)] ⁺	3.84E+05	7.85	203
26Cu	712.19	[Cu(Teb)₂Cl]⁺	9.74E+05	19.91	137

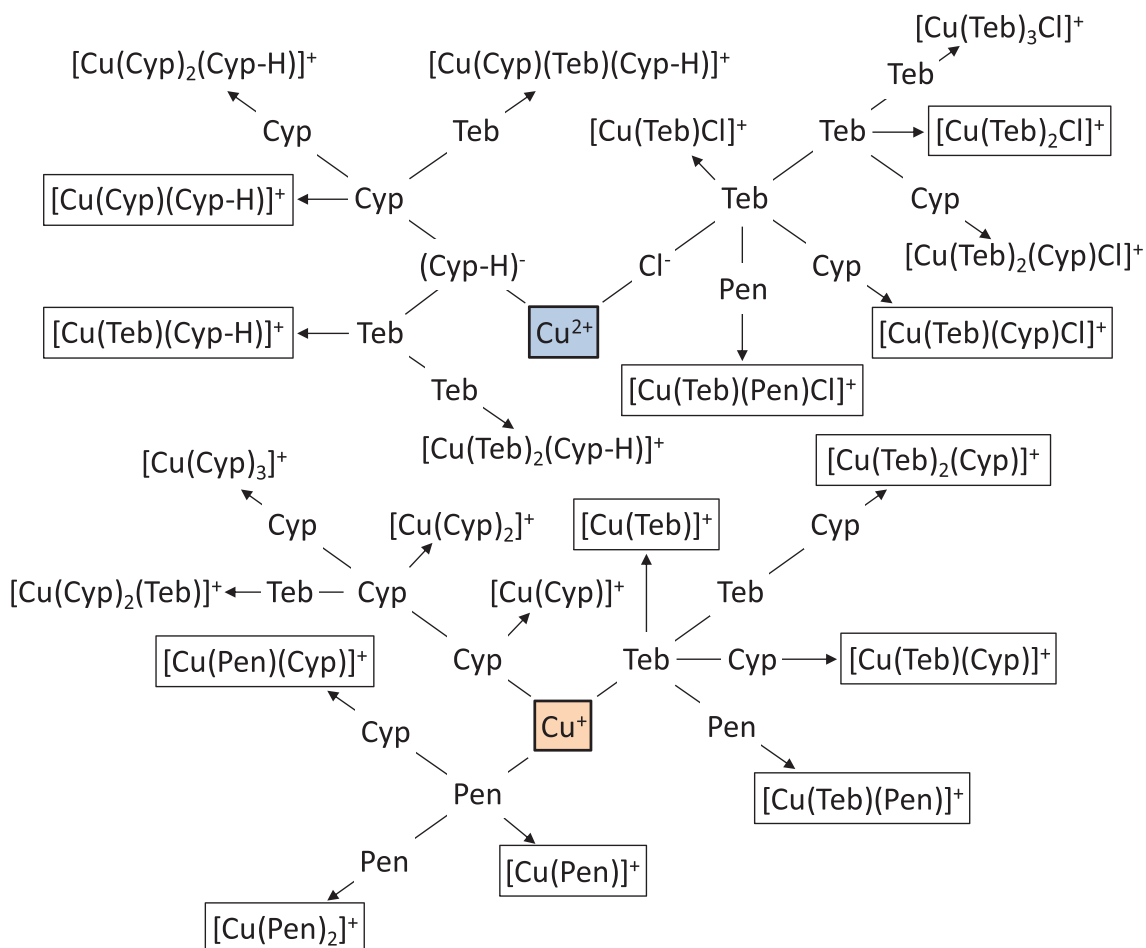


Fig. 2. Generative system of Cu^{2+} and Cu^+ complexes with penconazole (Pen), cyproconazole (Cyp), tebuconazole (Teb).

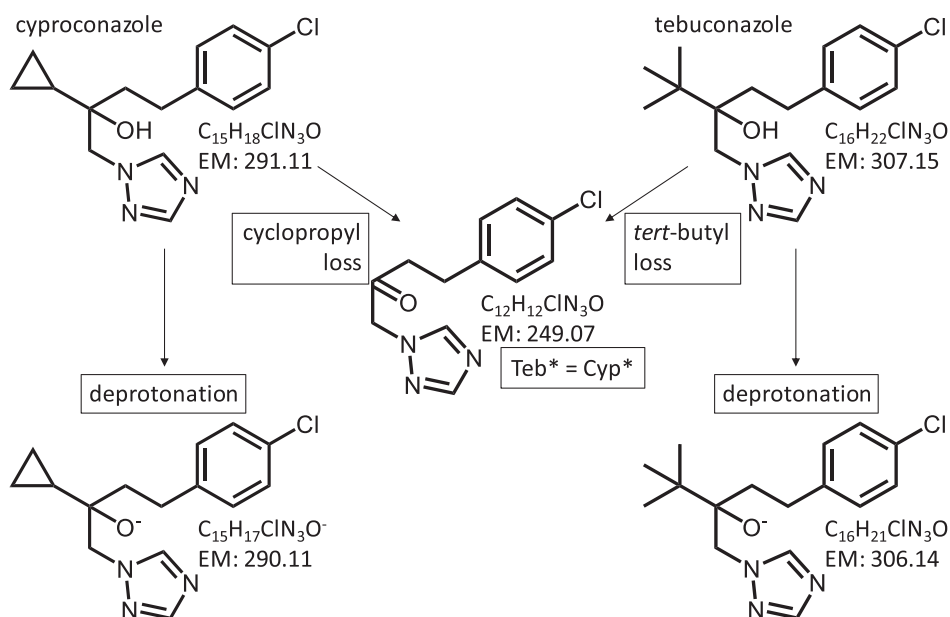


Fig. 3. Metabolites/degradation products in the presence of Cu coming from cyproconazole (Cyp) and tebuconazole (Teb) through deprotonation or the loss of tert-butyl or cyclopropyl moiety (Cyp*, Teb*).

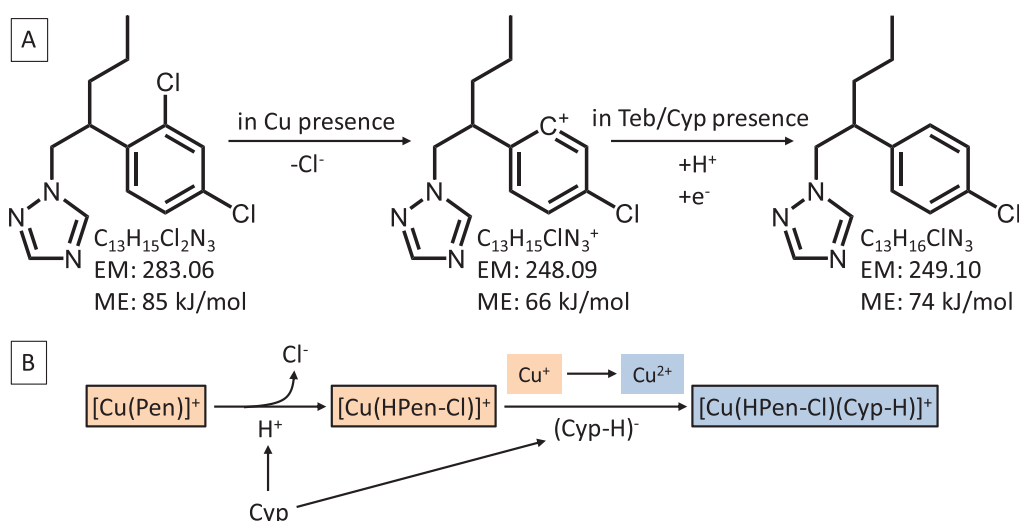


Fig. 4. A. Transformation of penconazole in the presence of Cu^{2+} and tebuconazole (**Teb**) or cyproconazole (**Cyp**). EM is exact mass and ME is the minimum energy calculated using molecular dynamics (Chem3D Pro 19.0, PerkinElmer Informatics). B. Transformation of the penconazole (**Pen**) complex with copper in the presence of **Cyp**.

dications via the 4N of the triazole ring, via the π -electrons of the chlorophenyl ring and via the oxygen atom of the OH group. The creation of this type of bonds also explains the deprotonation of **Cyp** (Jakl et al., 2017) and **Pen** (Kovač et al., 2020).

The strong tendency to create stable complexes with dications can be taken to advantage in triazoles analysis in environmental matrices (Jaklová Dyrtrtová et al., 2016; Jaklová Dyrtrtová et al., 2013). On the other hand, we have taken into account that this tendency significantly influences the reactivity of triazoles (Jaklová Dyrtrtová et al., 2018).

The experimental aromatase activity in cocktails (copper ions and triazoles together) was at least 16% lower than hypothetically expected. The highest aromatase decrease effect occurred in the cocktails containing **Pen** (more than 35%). The system consisting of Cu^{2+} and **Pen** was described in detail in Kovač et al. (2020). The most important information on this system here is the creation of **Pen** metabolites. The presence of copper ions induces the loss of one chlorine from **Pen** to create $(Pen-Cl)^+$ with m/z 248⁺, and the loss of two hydrogens, which could be explained by the creation of a double bond or an additional ring (Kovač et al., 2020; Schwack and Hartmann, 1994).

The most complex system is **Teb/Pen/Cyp/Cu²⁺**, which contains all the possible adducts, complexes as well as degradation products (Fig. 1). Other spectra of each particular systems are shown in the supplementary material.

The spectrum received from the **Teb/Pen/Cyp/Cu²⁺** system (Fig. 1) mostly contains Cu^+ complexes. The most abundant as well as the most stable (based on AE values) are $[Cu(Teb)_2Cl]^+$, $[Cu(Teb)(Pen)(H_2O)Cl]^+$, $[Cu(Teb)(Pen)Cl]^+$, $[Cu(Pen)(Pen-Cl)]^+$, $[Cu(Teb^*)]^+$ and $[Cu(Teb)(Teb^*)]^+$. Selected CID spectra of these mass-selected ions together with their AEs are shown in the supplementary material (Fig. S15). The complete image of the Cu^{2+}/Cu^+ , **Teb**, **Pen** and **Cyp** complexes/adducts listed in Table 2 is depicted in Fig. 2.

The most important complexes created from copper cations with **Teb**, **Cyp** and **Pen** are summarised in Fig. 2. Copper is present in the complexes as Cu^{2+} , where the negative charge coming from Cl^- or deprotonated **Cyp** or **Teb** (Fig. 3) has a stabilizing effect on the higher oxidation state of copper. Some part of the Cu^{2+} state has also been reduced in the electrospray to Cu^+ . The preservation of the Cu^{2+} requires the stabilizing impact of a negatively-charged

counter-ion or at least four neutral ligands (Jaklová Dyrtrtová et al., 2011; Tintaru et al., 2009).

Fig. 2 shows the genealogy of the generation of complexes. It is not a reaction scheme, only an explanation of which $Cu^{2+/+}$ complexes are created. Theoretically, all the statistical combinations are possible, but some of the complexes are preferred more than others. These preferences might be explained by high AEs of those complexes as well as by their abundance in the spectra (Table 2).

In the presence of Cu^{2+} several types of **Cyp**, **Teb** (Fig. 3) and **Pen** degradation products are created. Namely: (i) **Pen** loses its chlorine atom (details in (Kovač et al., 2020)), (ii) **Teb** loses its *tert*-butyl group (also observed in (Jakl et al., 2017; Jaklová Dyrtrtová et al., 2013)) (Fig. 3), and **Cyp** loses its isopropyl group (Jakl et al., 2017) (Fig. 3) to receive the same metabolite as from **Teb**.

For the **Teb/Pen/Cu²⁺** cocktail the experimental aromatase activity was almost about 41% lower than expected. The mass spectrum in the positive-ion mode is depicted in Fig. S11. It shows the same complexes, as those that can be seen in the spectrum received from the system consisting of **Teb/Pen/Cyp/Cu²⁺** (Fig. 1). Of course, the complexes/adducts containing **Cyp** are not present. However, the spectrum of **Teb/Pen/Cu²⁺** (Fig. S11) contains three complexes $[Cu(Teb)(Teb^*)]^+$, $[Cu(Teb)(Teb-H)]^+$, and $[Cu(Teb)_2]^+$ that are not present in the **Teb/Pen/Cyp/Cu²⁺** mixture (Fig. 1). These complexes contain $Cu^{2+/+}$ and **Teb** only and their abundance in the spectrum (Fig. S11) is not high. Only $[Cu(Teb)(Teb^*)]^+$ appears in a relatively high amount (4.04% of the total signal of all the complexes). These complexes are not abundant in the **Teb/Pen/Cyp/Cu²⁺** mixture (Fig. 1) probably because of their thermodynamic competition with more stable complexes.

The spectrum of the **Pen/Cyp/Cu²⁺** system (Fig. S12) contains the expected complexes/adducts, which are present also in **Teb/Pen/Cyp/Cu²⁺** (Fig. 1). The system of **Teb/Cyp/Cu²⁺** is shown in Fig. S14.

A detailed comparison of the spectra of different cocktails with Cu has revealed interesting facts. First of all, the signal with m/z 312.00 is attributable to two cations, $[Cu(Teb^*)]^+$ and $[Cu(Cyp^*)]^+$, because **Cyp*** and **Teb*** are the same fragments ($C_{12}H_{12}ClN_3O$, Fig. 3). Additionally, there is $[Cu(HPen-Cl)]^+$, where **HPen-Cl** is a neutral fragment transformed from the $(Pen-Cl)^+$ ion created in Cu ions presence (Kovač et al., 2020) by **Teb** or **Cyp** (Fig. 4). It means the catalysis of Cl^- cleavage by Cu^{2+} leads to $C_{13}H_{15}ClN_3^+$ formation, which is relatively unstable, and in the presence of H^+ , it is

transformed into neutral $C_{13}H_{16}ClN_3$. The 3D structures of Pen metabolites are depicted in Fig. S16. In this case, the source of H^+ is **Teb** or **Cyp**; in liquids, however, it can be something more willing to release a proton (e.g. H_2O). Triazoles are considered to be weak bases with pK_a around 1.5 (Konášová et al., 2015).

The completion of this redox reaction requires an electron. There is only one candidate to give electrons (be oxidised) – it is Cu^+ . Cu^+ is easily created from Cu^{2+} in electrospray in the presence of **Teb** (Jaklová Dyrtrtová et al., 2011) as well as **Cyp** (Jakl et al., 2017). This one-electron reduction of copper ion under electrospray conditions is explained in detail in (Agrawal and Schröder, 2011; Schröder, 2004). The first step of the **Pen**/ Cu reaction is just a simple decay (the loss of Cl^-). In the second step, $(Pen-Cl)^+$ gains one H^+ , after which it gains an e^- from Cu^+ . This redox reaction is also confirmed in the spectra (Figs. 1 and 4A), where all the participants of the redox reaction are present. The $Cu^{2+/+}$ system is considered to be a radical induction system (Jaklová Dyrtrtová et al., 2018), known as a Fenton reaction. In real environmental systems, where the cocktail of the present chemicals is richer and more complicated than our triazolic cocktails, the risk of the generation of free radicals is more reasonable. Additionally, the presence of triazoles in the system has the quenching effect on the $Cu^{2+/+}$ system (Jaklová Dyrtrtová et al., 2018).

We have demonstrated that the interaction of triazoles and metals is a complex process and its impact on metabolism seems to be rather extensive and must be evaluated in the context of biochemical reactions *in vivo*.

4. Conclusion

The group effects did not differ significantly from the calculated average of the effects of every particular triazoles in a mixture. This means that the reactivity of triazoles in groups is not significantly affected by interactions among them. On the other hand, the presence of copper together with triazole(s) (cocktail effect) considerably increases the inhibitory effect to aromatase activity. The highest inhibitory effect (greater than 60%) has been observed in the mixtures containing penconazole with Cu . The presence of copper in ionic forms is crucial for the formation of triazole metabolites. Namely, tebuconazole loses the *tert*-butyl group, cyproconazole loses the cyclopropyl group, and penconazole loses one chloride anion. Additionally, although the triazoles are considered to be weak bases, in the presence of Cu ions they deprotonate.

Under electrospray conditions in the presence of triazoles, Cu^{2+} is reduced to Cu^+ . Cu^+ is known to initiate the Fenton reaction, which leads to radical production. However, in the presence of triazoles (mostly penconazole) Cu^+ is oxidised back to Cu^{2+} . Therefore, triazoles can be considered as possible quenchers of this copper Fenton reaction.

Evidently, the situation is really complicated even in the case of several possibly reactive members (e.g. Cu^{2+} , penconazole and cyproconazole). Further, it is essential to predict the real impact of an applied treatment and to include all possibly important parts of the system.

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CRediT authorship contribution statement

Jana Jaklová Dyrtrtová: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Funding acquisition. **Kateřina Bělonožníková:** Investigation, Formal

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Declaration of competing interest

The authors declare that there are no competing interests associated with the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2020.115201>.

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