



Organic compounds as Photosystem II inhibitors and Cu²⁺ ligands: insights from primary screening

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Abstract

The search for novel organic compounds as PSII inhibitors and copper (Cu²⁺) ligands addresses key agricultural challenges. Three novel compounds were synthesized and evaluated for PSII photochemical activity inhibition and Cu²⁺-chelating ability. Primary screening was performed by recording effects on minimal and maximal chlorophyll fluorescence levels in isolated thylakoids. Testing employed both direct (thylakoids + Cu²⁺ + compound) and reverse (thylakoids + compound + Cu²⁺) addition sequences, with measurements performed under short illumination (<10 s) to avoid photodynamic artifacts. The tested compounds showed no pronounced inhibitory activity toward PSII photochemistry and exhibited no strong Cu-chelating capacity. Nevertheless, these data enable formulation of more stringent structural requirements for candidate molecules and confirm the high information content of the proposed combined screening approach for further biologically active substance design.

Keywords: chlorophyll fluorescence; copper ions; ligands; novel organic compounds; photosystem II; thylakoids.

Introduction

The search for and investigation of novel tested organic compounds (TOCs) – potential inhibitors of PSII and

ligands for Cu²⁺ ions – is driven by the persistent need to increase yields of economically important crops. Practical implementation of this task is progressing along two key directions: creation of highly effective herbicidal

Highlights

- Combined fluorescence screen for PSII inhibitors and copper chelators
- Testing three novel compounds defined structural requirements for candidates
- Proposed approach is highly informative for designing new bioactive compounds

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Abbreviations: AL – actinic light; EDTA – ethylenediaminetetraacetic acid; EGTA – ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid; F₀ – minimal (dark-adapted) chlorophyll fluorescence level of PSII; F_m – maximal chlorophyll fluorescence level of PSII; TOC – tested organic compound.

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preparations that suppress weed vegetation by inhibiting photosynthesis, and development of chelate systems for targeted delivery of micronutrients to optimize plant mineral nutrition (Rodionova *et al.* 2017, Madhupriya *et al.* 2024, Umurzokov *et al.* 2025). The rapid evolution of weed resistance to traditional preparations stimulates the search for new compounds (Rodionova *et al.* 2017, Umurzokov *et al.* 2025). Chelation of micronutrients with organic molecules increases their stability, assimilation, and translocation in plants, mitigating deficiencies that reduce crop yields (Rodionova *et al.* 2017, Madhupriya *et al.* 2024).

TOCs belong to a broad class of nitrogen- and sulfur-containing heterocyclic molecules, which are widely recognized in medicinal chemistry and agrochemistry for their diverse biological activities. Compounds with similar fused heterocyclic cores or acylthiourea fragments are known to exhibit various bioactivities, including antimicrobial, antioxidant, and, in some specific cases, interactions with the photosynthetic apparatus or the ability to coordinate transition metal ions. The structural versatility of these scaffolds makes them promising candidates for dual-purpose screening.

To be an effective inhibitor of PSII electron transport (similar to atrazine or diuron), a compound must: have a low molecular mass (up to 300 Da; for atrazine – 215.68, diuron – 233.09) to physically fit into the volume of the Q_B -binding pocket of the D1 protein (Battagliana *et al.* 2021, Wang *et al.* 2022); possess moderate lipophilicity ($\log P$ 2.0–4.0; for atrazine and diuron 2.5–2.8) for effective membrane permeability and accumulation in the thylakoid membrane (Fedtke 1982, Hess 2000); the molecule must remain neutral at the physiological pH of the stroma (pK_a outside the 5–9 range) to ensure passive diffusion (Tischer and Strotmann 1977); structurally, it must contain a flat or slightly non-planar heterocyclic or aromatic core (triazine, phenylurea, pyrimidine, *etc.*) containing atoms capable of acting as strict hydrogen bond donors and acceptors for interaction with Ser264 and His215 of the D1 protein; and it must necessarily possess specific hydrophobic substituents (halogens, alkyl, or aryl groups) critical for filling the hydrophobic sub-pockets of the Q_B site and providing van der Waals contacts that stabilize the inhibitor–receptor complex.

A large body of scientific literature convincingly demonstrates that Cu^{2+} causes a dose-dependent decrease in the maximal level of PSII chlorophyll fluorescence – the F_m parameter – which closely correlates with the suppression of its photochemical activity. This effect is stably recorded both *in vivo* on intact leaves (Ouzounidou 1996) and *Chlorella pyrenoidosa* cells (Vavilin *et al.* 1995), as well as *in vitro* on isolated chloroplasts, thylakoid membranes, and PSII particles. The degree of F_m decrease is strictly proportional to the toxicant concentration and critically depends on the buffer composition (Hsu and Lee 1988, Mohanty *et al.* 1989, Renganathan and Bose 1990, Renger *et al.* 1993, Boucher and Carpentier 1999, Dewez *et al.* 2007). To date, it is generally accepted that there are at least two fundamentally distinct mechanisms of Cu^{2+} toxic action on PSII: "dark" (direct) and "light" (indirect).

The dark mechanism manifests itself through direct binding of Cu^{2+} to PSII components, leading to structural and functional impairments of the complex regardless of light presence. However, to instrumentally measure the decrease in F_m , the sample must be illuminated with actinic light (AL) to close the reaction centers. At this point, the light mechanism of toxicity comes into play: the presence of Cu^{2+} makes the PSII complex extremely vulnerable to photoinhibition (Yruela *et al.* 1996, Päsikkä *et al.* 1998, 2001, 2002). Consequently, if Cu-treated PSII samples are illuminated for more than 30 seconds, a rapid additional drop in F_m begins, driven not by the primary dark effect, but by secondary light-dependent mechanisms (Arellano *et al.* 1995). Therefore, to correctly assess the degree of F_m decrease caused exclusively by the dark action of Cu^{2+} ions, the illumination of PSII for F_m recording must be very brief (no more than 30 s, or, if experimental conditions permit, a 0.8–1 s saturating flash is sufficient).

It has been experimentally shown (Arellano *et al.* 1994, Barón *et al.* 1995, Pádua *et al.* 2010) that the inhibition of PSII by Cu^{2+} ions can be reversed by adding chelating agents (such as EDTA), but only before irreversible structural damage occurs. Moreover, to guarantee the displacement of non-specifically bound Cu^{2+} from PSII preparations, millimolar concentrations (5 mM, *i.e.*, in molar excess) of the chelating agent EDTA are standardly used (Arellano *et al.* 1994, Barón *et al.* 1995, Pádua *et al.* 2010).

For newly synthesized organic compounds to act as effective chelators capable of extracting Cu^{2+} ions from thylakoid membranes and PSII, they must possess a specific synergistic combination of physicochemical properties. Relying on the mechanism of action of reference chelators (EDTA, EGTA), a profile guaranteeing such activity can be formulated for preliminary theoretical analysis (Ćendić *et al.* 2017, Persson *et al.* 2020, Tosato *et al.* 2021, Sajjadi *et al.* 2024, Zonzin *et al.* 2025). The fundamental basis is a high thermodynamic stability constant ($\log K$), creating the necessary gradient to displace the metal from the biocomplex, which must be combined with an optimal molecular mass (EDTA – 292 Da, EGTA – 380 Da) and compactness to ensure kinetic diffusion to hidden binding sites. Mandatory conditions include high hydrophilicity and a favorable acidity constant (pK_a) profile, ensuring ligand deprotonation and the availability of donor groups at physiological pH values. In addition, the molecule must possess conformational flexibility to realize the entropically favorable chelate effect and contain specific donor atoms (nitrogen and oxygen) selective for the Cu^{2+} ion as a "borderline" Lewis acid (Ćendić *et al.* 2017, Persson *et al.* 2020, Tosato *et al.* 2021, Sajjadi *et al.* 2024, Zonzin *et al.* 2025).

Primary screening of the synthesized compounds as potential inhibitors of PSII photochemical activity was conducted by recording their effects on the minimal (dark, F_0) and maximal (F_m) levels of chlorophyll fluorescence. The choice of method is due to the fact that the recording of fluorescence parameters (in particular, using PAM fluorometry) is one of the most rapid, highly informative,

and noninvasive methods for assessing the functional state of the photosynthetic apparatus (Kalaji *et al.* 2017, Guidi *et al.* 2019). According to modern concepts, the F_0 level serves as a highly sensitive indicator of the structural integrity of the light-harvesting antenna complexes and the oxygen-evolving complex, whereas the F_m value directly reflects the functional activity of the reaction centers and the degree of reduction of the primary quinone electron acceptor (Q_A). A comprehensive analysis of the shifts in these basic parameters, as well as the derived parameter – the maximal quantum yield of PSII (F_v/F_m) – allows not only to reliably state the fact of suppression of linear electron transport but also to preliminarily localize the site of action of new substances in the electron transport chain of thylakoid membranes with high accuracy (Kalaji *et al.* 2017, Guidi *et al.* 2019). Nevertheless, it is important to acknowledge that the transition from F_0 to F_m involves complex kinetic mechanisms, and the direct strict equivalence of the F_v/F_m ratio to the quantum yield of photochemistry has been subject to nuanced discussion in the literature (Garab 2025).

The aim of this work was the primary screening of a series of novel organic compounds to identify those that exhibit the ability to suppress the photochemical activity of PSII (decrease F_m) in thylakoids and/or reverse the inhibitory effect of Cu^{2+} ions on the maximal chlorophyll fluorescence (F_m) in thylakoids *in vitro*.

Materials and methods

Tested organic compounds and their physicochemical properties: In this study, we used three novel synthesized compounds, hereafter referred to as tested organic compounds (TOC1, TOC2, and TOC3). The synthesis of these novel organic compounds was carried out as detailed previously (Tuck 1979). Stock solutions were prepared in dimethyl sulfoxide (DMSO). The final concentration of DMSO in all experimental samples did not exceed 1% (v/v) and did not affect chlorophyll fluorescence parameters, which was confirmed by control measurements with the corresponding solvent concentration without the tested compounds (data not shown).

Calculation of molecular descriptors (number of rotatable bonds, hydrogen bond donors and acceptors, number of rings and aromatic rings) for the tested compounds TOC1–TOC3, as well as for atrazine and diuron, was performed using the *PubChem* chemical database (National Center for Biotechnology Information, NCBI, USA; <https://pubchem.ncbi.nlm.nih.gov/>). Unique identifiers CID: 2256 and CID: 3120 were used to identify atrazine and diuron, respectively. Topological parameters were pre-calculated by the *Cactvs* cheminformatics algorithm (version 3.4.8.18, *Xemistry GmbH*) integrated into the *PubChem* platform. The acid-base properties of the tested compounds were evaluated by calculating pK_a using the *pKa Prediction* module of the *ChemAxon Marvin* software package (version 23.18, *ChemAxon Ltd.*, Budapest, Hungary; <https://www.chemaxon.com>) (accessed 14 August 2026). The calculation was performed for the most probable ionization centers at physiological

pH. The predicted pK_a values for the main centers were: for N-diethylthiocarbamoyl-1-ethyl-2-oxo-4-phenyl-1,2-dihydropyrimidine-5-carboxamide – 7.8 ± 0.3 ; for 1,3,5-triazino[1,2-a]benzimidazol-2-amine, 3,4-dihydro-4-thiophen-2-yl – 7.0 ± 0.2 ; for 1,3,5-triazino[1,2-a]benzimidazol-2-amine, 3,4-dihydro-4-(4-imidazole) – 6.7 ± 0.3 . Experimental verification of the pK_a values was not performed.

Thylakoid isolation: Thylakoids were isolated from leaves of greenhouse spinach (*Spinacia oleracea* L.) using a method similar to that described previously (Khorobrykh and Ivanov 2002), without significant modifications to the original protocol, and stored at -80°C until use.

Cu^{2+} ions were added as a $CuCl_2$ solution at a final concentration of 40 μM .

Measurement of PSII photochemical activity:

The photochemical activity of PSII was analyzed by measuring the photoinduced kinetics of changes in the PSII chlorophyll fluorescence yield associated with the photoreduction of the primary PSII electron acceptor, plastoquinone Q_A , using a pulse-amplitude-modulation (PAM) fluorimeter (*XE-PAM*, *Heinz Walz*, Germany) and the accompanying *Power Graph Professional 3.3* software in a 10-mm diameter cuvette at room temperature. The fluorescence level values before the application of actinic light (F_0 , measured under weak measuring light) and the maximal level F_m were determined. Before fluorescence measurements, the thylakoid samples were dark-adapted for 15 min to ensure the full oxidation of the primary quinone acceptor Q_A and the opening of all PSII reaction centers. To record F_0 , a sample pre-adapted to darkness was illuminated with weak probing pulses of measuring light ($\lambda = 490 \text{ nm}$; $4 \text{ m}^{-2} \text{ s}^{-1}$; xenon measuring flash lamp, 64 Hz, *BG39*, *Schott*). To record F_m , dark-adapted samples were illuminated with a saturating pulse of light [duration 0.8 s; $\lambda = 490 \text{ nm}$; $1,000 \mu\text{mol}(\text{photon}) \text{ m}^{-2} \text{ s}^{-1}$, *BG39*, *Schott*] (Zharmukhamedov *et al.* 2022). The overall photoinduced fluorescence kinetics were recorded continuously for up to 30 seconds depending on the specific experimental protocol.

Chlorophyll fluorescence measurements (F_m) were performed in a standard reaction buffer ensuring the stability of thylakoid membranes and PSII: 20 mM Tris-HCl (pH 7.7), 35 mM NaCl, and 10 mM $MgCl_2$, with the thylakoid concentration in terms of chlorophyll in all experiments, strictly controlled *via* spectrophotometric measurement of chlorophyll content according to standard protocols (Lichtenthaler and Buschmann 2001), equal to $10 \mu\text{g mL}^{-1}$. This buffer composition was chosen to maintain the native structure of the grana and prevent nonspecific protein denaturation, which allows evaluation of the ligand properties of the tested compounds under conditions as close to physiological as possible, including background competition from buffer components for Cu^{2+} ions (Schreiber 2004). Although it is known that Tris can weakly interact with Cu^{2+} (Renganathan and Bose 1990), at the concentration used (20 mM) and the Cu^{2+} concentration applied (40 μM), it does not completely

sequester the metal, allowing for the reliable observation of Cu^{2+} -induced F_m suppression (as confirmed by our control measurements).

Assay of Cu^{2+} chelating and inhibitory activity: Primary screening for potential inhibitory and chelating activity was conducted at a fixed Cu^{2+} concentration of 40 μM , based on the fact that in most publications, inhibitory Cu^{2+} concentrations range from 10 to 50 μM (Hsu and Lee 1988, Mohanty *et al.* 1989, Renger *et al.* 1993, Arellano *et al.* 1995, Yruela *et al.* 1996, Dewez *et al.* 2007).

To identify the ability of TOCs to act as Cu^{2+} ligands and reverse the inhibitory effect of Cu^{2+} , expressed as a decrease in F_m , TOCs were added at a final concentration of 2.0 mM. This concentration was chosen with a 50-fold molar excess relative to the inhibitory concentration of Cu^{2+} (40 μM). At such a molar excess of the chelating agent, it can be expected that the TOC competes for Cu^{2+} ions not only in solution but also displaces them from nonspecific binding centers on the membrane, which corresponds to the methodological approaches proven effective for the complete displacement of nonspecifically bound Cu^{2+} from thylakoid membranes using standard chelators such as EDTA (Arellano *et al.* 1994, Barón *et al.* 1995, Pádua *et al.* 2010).

In the primary screening of new TOCs for the ability to bind Cu^{2+} ions, the dark incubation time in the presence of TOC was 5 min in both direct and reverse addition orders. It is known that the chelation reaction of Cu^{2+} ions by macrocyclic and polydentate ligands (such as EDTA) proceeds almost instantaneously, reaching the diffusion limit and falling within the sub-millisecond range (Kirschenbaum and Kustin 1970, Oliwa and Orlik 2021). Experimentally, even in an artificially slowed model system (EDTA transfer from Ca^{2+} to Cu^{2+} ions in buffer at pH 6.5), the half-life of the reaction is only ~50 ms (Schroeter *et al.* 2022). Consequently, a five-minute dark incubation creates a substantial (more than 300,000-fold) time excess, guaranteeing the achievement of full thermodynamic equilibrium of the complexation reaction long before fluorescence recording begins.

Statistical analysis: All operations for the direct preparation of samples for incubation and measurement were performed in a specially equipped dark room under dim scattered green light [$\lambda \approx 520\text{--}550\text{ nm}$, intensity $<1\text{ }\mu\text{mol}(\text{photon})\text{ m}^{-2}\text{ s}^{-1}$], which excluded undesirable photoactivation and/or photoinhibition. Each experiment was performed in three biological and five technical (experimental) replicates. Results are presented as mean $\pm$ SE (standard error of the mean) or SD (standard deviation). The statistical significance of differences was evaluated using *Student's t*-test; the significance level was set at $p < 0.05$. Data processing was performed in *Origin 2021* (OriginLab Corporation).

Results and discussion

Physicochemical properties and primary theoretical screening of novel TOCs: Conducting a primary theoretical screening of novel organic compounds before

starting experimental research is a scientifically sound stage that allows assessing the probability of target properties based on analysis of structural and functional features of molecules. Such an approach does not replace the experiment and does not give a conclusion on the presence or absence of biological activity, but it allows one to: (a) identify structural fragments potentially relevant for interaction with the biological target; (b) evaluate physicochemical parameters affecting bioavailability and binding; (c) rank compounds by priority for subsequent experimental testing; (d) formulate specific testable hypotheses. Within this work, a theoretical analysis of three novel tested organic compounds (TOC1–TOC3, Table 1) was carried out from the perspective of their potential ability to: (1) inhibit electron transfer in PSII *via* a mechanism similar to the known herbicides atrazine and diuron; (2) chelate Cu^{2+} ions in the manner of the reference polyaminocarboxylate chelators EDTA and EGTA.

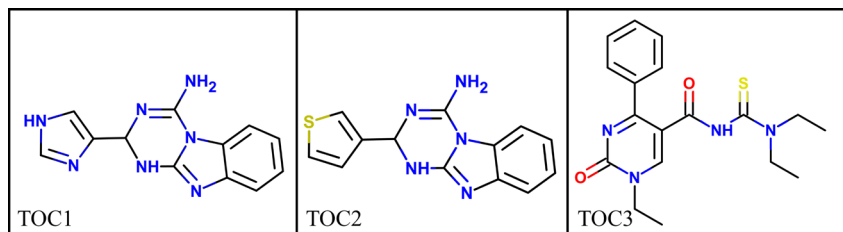
Descriptors were calculated to assess physicochemical properties, membrane permeability, and target affinity. An increase in molecular mass (MW), topological polar surface area (TPSA), the number of hydrogen bond donors/acceptors, and rotatable bonds beyond optimal values (Lipinski's rule of five: $\text{MW} < 500$, $\text{TPSA} < 140\text{ }\text{\AA}^2$, $\text{HBD} < 5$, $\text{HBA} < 10$, $\text{RotB} < 10$) reduces passive membrane permeability and bioavailability. An increase in lipophilicity (LogP) improves penetration through lipid membranes (which is critical for access to thylakoids); however, values above the optimum (1.0–5.0) sharply worsen aqueous solubility. An increase in the number of rings and aromatic rings increases the rigidity and planarity of the molecule, which improves complementarity when binding to flat hydrophobic pockets of targets (*e.g.*, the PSII Q_B site), but exceeding optimal values (1–3 rings, of which 1–2 are aromatic) reduces solubility and increases the risk of off-target toxicity.

Known PSII inhibitors – diuron (DCMU, phenylurea type) and atrazine (triazine type) – compete with plastoquinone for the Q_B -binding pocket of the D1 protein, blocking electron transfer from Q_A to Q_B . Key structural requirements for effective inhibition are: the presence of a hydrophobic aromatic fragment occupying the hydrophobic region of the pocket; donor–acceptor centers for the formation of hydrogen bonds with D1 protein residues (Ser264, His215, Phe265); moderate lipophilicity ($\log P \approx 1\text{--}4$) for penetration to the thylakoid membranes; and a neutral form at physiological pH. Diuron contains a dichlorophenyl hydrophobic block and a urea pharmacophore $-\text{NH}-\text{CO}-\text{NH}-$; atrazine contains an s-triazine ring with a chloro-substituent and alkylamino groups.

Structural analysis of TOC1–TOC3 (Table 1) indicates that all three compounds contain nitrogen-containing heterocyclic cores capable of hydrogen bonding, and aromatic fragments potentially providing hydrophobic interactions. TOC1 ($\text{C}_{13}\text{H}_{11}\text{N}_5\text{S}$, $M = 269.3\text{ g mol}^{-1}$) contains a thiophene substituent, increasing lipophilicity compared to the imidazole analog, and a triazino[1,2-*a*]benzimidazole core with a 2-amino group. TOC2 ($\text{C}_{12}\text{H}_{11}\text{N}_7$, $M \approx 253\text{ g mol}^{-1}$) differs by an imidazole substituent, which increases

Table 1. (A) Structural formulas of the tested organic compounds (TOC1–TOC3) with the systematic name according to IUPAC rules: TOC1 – 1,3,5-triazino[1,2-a]benzimidazol-2-amine, 3,4-dihydro-4-thiophen-2-yl; TOC2 – 1,3,5-triazino[1,2-a]benzimidazol-2-amine, 3,4-dihydro-4-(1H-imidazol-4-yl); TOC3 – N-diethylcarbamothioyl-1-ethyl-2-oxo-4-phenyl-1,2-dihydropyrimidine-5-carboxamide; (B) some physicochemical parameters of TOC1–TOC3. Asterisks (*, **) indicate reference compounds used for structural comparison. *National Center for Biotechnology Information (2026). *PubChem* Compound Summary for CID 2256, Atrazine. Retrieved August 13, 2026 from <https://pubchem.ncbi.nlm.nih.gov/compound/Atrazine>. **National Center for Biotechnology Information (2026). *PubChem* Compound Summary for CID 3120, Diuron. Retrieved August 13, 2026 from <https://pubchem.ncbi.nlm.nih.gov/compound/Diuron>. MW – molecular weight; LogP – octanol–water partition coefficient (lipophilicity); TPSA – topological polar surface area; pKa – acid dissociation constant; Rotatable bonds – number of rotatable bonds; H-bond donors – number of hydrogen bond donors; H-bond acceptors – number of hydrogen bond acceptors; Ring count – total number of rings; Aromatic ring count – number of aromatic rings.

A



B

	MW [g mol ⁻¹]	LogP	TPSA [Å ²]	pK _a	Rotatable bonds	H-bond donors	H-bond acceptors	Ring count	Aromatic ring count
TOC1	269.33	2.38	68.23	6.7 ± 0.3	1	2	5	4	3
TOC2	253.27	1.05	96.91	7.0 ± 0.2	1	3	5	4	3
TOC3	358.47	2.29	67.23	7.8 ± 0.3	5	1	4	2	2
Atrazine*	215.68	2.60	62.70	1.6	4	2	5	1	1
Diuron**	233.09	2.68	32.30	13.2	1	1	1	1	1

polarity and the probability of protonation at neutral pH. TOC3 (C₁₈H₂₂N₄O₂S, M ≈ 358.5 g mol⁻¹) contains an acylthiourea fragment –C(=O)–NH–C(=S)–N(C₂H₅)₂, formally resembling the urea pharmacophore of diuron, as well as a phenyl hydrophobic substituent and diethyl groups.

At the same time, none of the compounds fully reproduces the pharmacophore of classical inhibitors. TOC1 and TOC2 lack the urea fragment and pronounced lipophilic substituents (chlorophenyl, isopropylamino); their condensed polycyclic systems differ in geometry from the compact structures of atrazine and diuron. TOC3, despite having a fragment remotely resembling urea, has a different topology of pharmacophoric centers: the heteroaryl fragment is attached *via* a carbonyl carbon, not *via* nitrogen, as in classical phenylureas. In addition, the thiocarbonyl group C=S differs from the carbonyl C=O in size, polarizability, and ability to form hydrogen bonds.

Conclusion on PSII inhibition: TOC1 is characterized as a potential weak/moderate candidate due to the presence of a thiophene hydrophobic fragment and a triazine core; TOC2 is the least likely candidate due to high polarity and a tendency to protonate; TOC3 is the most structurally similar to the urea pharmacophore, but its geometry and increased size create significant uncertainty. None of the compounds can be qualified as a classical PSII inhibitor without experimental confirmation.

Reference chelators EDTA and EGTA are polyaminocarboxylate ligands that bind Cu²⁺ through 5–6

donor atoms (2 N atoms and 3–4 carboxylate O atoms) to form thermodynamically highly stable complexes (logK for Cu–EDTA ≈ 18). Key features: high denticity, polyanionic charge, backbone flexibility, pronounced chelate effect, and good water solubility.

All three TOC1–TOC3 compounds contain potential nitrogen and/or sulfur donor atoms capable of coordinating Cu²⁺. TOC1 has nitrogen centers of the triazine and benzimidazole fragments, as well as thiophene sulfur; however, thiophene sulfur is a weak donor due to the involvement of the lone pair in the aromatic sextet. TOC2 contains an imidazole fragment – a known coordination center for Cu²⁺ (by analogy with histidine in copper-containing proteins), as well as multiple nitrogen centers; however, the rigid geometry of the molecule limits the possibility of forming a stable chelate cycle. TOC3 possesses thiocarbonyl sulfur C=S, two carbonyl oxygens, and a deprotonable NH center, which theoretically allows bidentate coordination (S,O or S,N after deprotonation); at the same time, the thiourea fragment may exhibit redox activity towards Cu²⁺.

The fundamental differences of all three compounds from EDTA/EGTA are as follows: the absence of carboxylate groups and polyanionic charge; low denticity (actually 1–3 donor atoms vs. 5–6); a rigid or partially rigid structure (as indicated by the ring count, Table 1) preventing complete "enclosure" of the metal; limited water solubility (reflected by LogP and TPSA values, Table 1); and a neutral or weakly ionized character at physiological pH.

Conclusion on Cu^{2+} chelation: TOC1 can be considered a moderate N,S-donor ligand; TOC2 – as a potentially more effective N-donor ligand due to the imidazole fragment; TOC3 – as a ligand involving thiocarbonyl sulfur and carbonyl oxygens, but with a risk of redox interactions. None of the compounds is an analog of EDTA or EGTA in terms of denticity, charge, and complex stability.

The conducted theoretical analysis, based on the structural and physicochemical descriptors (Table 1), allows us to conclude that all three TOC1–TOC3 compounds contain separate structural elements potentially relevant both for PSII inhibition and for Cu^{2+} coordination, but none of them fully reproduces the pharmacophoric and physicochemical characteristics of reference compounds. The expected activity, if detected experimentally, is most likely to be weak or moderate. Given the fundamental structural and physicochemical differences between the tested compounds and reference chelators like EDTA/EGTA, the current experimental work is strictly focused on the primary screening and comparative analysis of the three novel TOCs with each other, rather than benchmarking them against classical synthetic chelators.

To experimentally verify the predictions formulated based on theoretical analysis, a primary screening of the effect of TOC1–TOC3 on the chlorophyll fluorescence parameters of PSII in isolated spinach thylakoids was conducted.

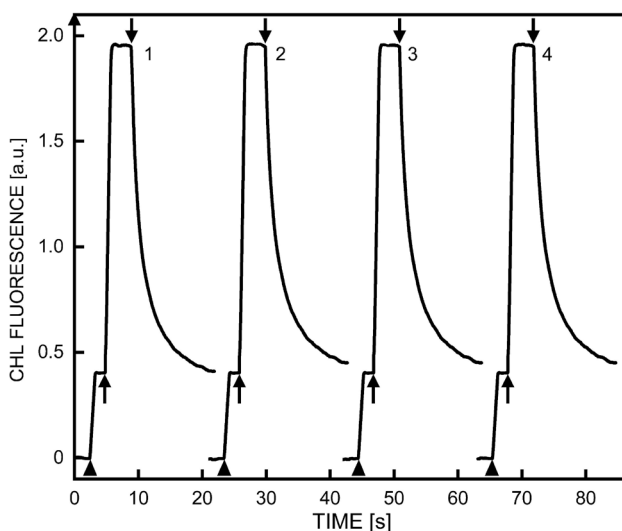


Fig. 1. Kinetics of photoinduced changes in the PSII chlorophyll fluorescence yield measured on spinach thylakoids in the absence of other additives (kinetics 1) and in the presence of the TOC1 compound at a concentration of 2.0 mM (kinetics 2–4), after 5 min (kinetics 2), 10 min (kinetics 3), 17 min (kinetics 4) of dark incubation with stirring. Chlorophyll concentration is $10 \mu\text{g mL}^{-1}$. Triangles indicate the moments of switching on the measuring light ($\lambda = 490 \text{ nm}$; $2 \mu\text{mol}(\text{photon}) \text{ m}^{-2} \text{ s}^{-1}$; xenon measuring flash lamp, 64 Hz, BG39, Schott). Upward and downward arrows indicate the moments of switching on and off the actinic light, respectively ($\lambda = 490 \text{ nm}$; $1,000 \mu\text{mol}(\text{photon}) \text{ m}^{-2} \text{ s}^{-1}$, BG39, Schott).

Primary screening of PSII inhibitory properties of TOCs: Fig. 1 shows the typical kinetics of photoinduced changes in the chlorophyll fluorescence yield (F_v) of thylakoids, measured in the presence of 2.0 mM TOC1 (kinetics 2–4, respectively) in comparison with the control (kinetics 1) after dark incubation of thylakoids in the presence of TOC1 for 5, 10, and 17 min. The primary screening of novel organic compounds (TOC1–TOC3) for their ability to inhibit PSII photochemical activity was based on assessing their effect on the basic fluorescence parameters: minimal (F_0) and maximal (F_m) levels, the variable fluorescence value (F_v), as well as the kinetics of the dark decay of F_m , reflecting the rate of reoxidation of the primary quinone acceptor Q_A^- . As shown in Fig. 1, the addition of TOC1 did not induce any significant shifts in the basal photochemical parameters of PSII. The preservation of the F_v/F_m ratio and the unchanged dark relaxation kinetics of F_v indicate that the compound neither disrupts the structural integrity of the light-harvesting complexes nor blocks the electron transport chain at the acceptor side of PSII, unlike classical Q_B -site inhibitors.

To ensure the reliability of the screening, experiments were performed on intact, highly active thylakoids. The functional integrity of the control samples (Fig. 1, curve 1) is confirmed by the reproducibility of the absolute fluorescence values: $F_0 = 0.40 \pm 0.04 \text{ rel. units}$ and $F_m = 1.96 \pm 0.18 \text{ rel. units}$ (mean $\pm$ SE, $n = 5$), which also indicates the stability of the hardware settings. The dimensionless coefficients calculated on this basis were $F_v/F_m = 0.80 \pm 0.03$ and $F_v/F_0 = 3.89$. These indicators fully correspond to the generally accepted physiological range for healthy isolated thylakoids, for which the maximal quantum yield of PSII (F_v/F_m) varies in the range of 0.75–0.85, and the F_v/F_0 ratio is 3.0–5.0 (Maxwell and Johnson 2000, Murchie and Lawson 2013, Jiao and Hu 2025). The obtained values are consistent with literature data; thus, in the work of Dhir *et al.* (2008), at an identical chlorophyll concentration ($10 \mu\text{g mL}^{-1}$), the control values were $F_0 = 350$ and $F_m = 1,650 \text{ rel. units}$, giving $F_v/F_m = 0.78$ and $F_v/F_0 = 3.71$. For the quantitative assessment of inhibitor effects, absolute values were normalized relative to the control group, whose photochemical activity was taken as 100%. The use of universal dimensionless ratios made it possible to minimize hardware errors and correctly compare the efficiency of PSII operation in different samples.

To ensure the reliability of the experimental data for the primary screening of the PSII-inhibiting properties of new TOCs, these compounds were used in these experiments at a concentration of 2.0 mM.

As can be seen from the presented data, the TOC1 compound at a concentration of 2.0 mM did not have a significant effect on either the F_m and F_0 levels, the variable fluorescence value F_v ($F_v = F_m - F_0$), or the rate of F_m relaxation in the dark, and this effect did not depend on the dark incubation time. In the presence of 2.0 mM TOC1 after 5 min of dark incubation, the values were: $F_0 = 0.41 \pm 0.05 \text{ rel. units}$, $F_m = 1.94 \pm 0.21 \text{ rel. units}$, $F_v/F_m = 0.79 \pm 0.04$ ($n = 5$, $p > 0.05$ compared to control).

Similar results were obtained for TOC2 and TOC3 (data not shown).

The maintenance of the F_0 level at control values indicates that TOC1 did not cause uncoupling of the light-harvesting complexes from the PSII reaction centers and did not lead to the appearance of photochemically inactive centers. The absence of changes in the F_m and F_v values indicates the absence of blocking of the electron transport chain on the acceptor side of PSII (unlike the action of classical inhibitors, such as diuron, which bind to the Q_B site of the D1 protein). In addition, the unchanged rate of dark decay of F_v confirms that the compounds did not disrupt the processes of dark reoxidation of Q_A^- by the plastoquinone pool. Absolutely identical results were obtained for the rest of the compounds from the primary screening series (TOC2 and TOC3): none of the studied substances caused a decrease in F_m and F_v , did not change the F_0 level, and did not affect the rate of the dark decay of fluorescence (data not shown separately). Thus, the results of the primary screening allow us to conclude that the studied classes of organic compounds do not exhibit properties characteristic of direct inhibitors of PSII photochemical activity.

The next stage of the work was the experimental verification of the ability of TOC1–TOC3 to chelate Cu^{2+} ions, based on assessing their effect on Cu-induced suppression of F_m .

Primary screening of Cu^{2+} -chelating properties of TOCs: Preliminarily, we ensured that the intensity of

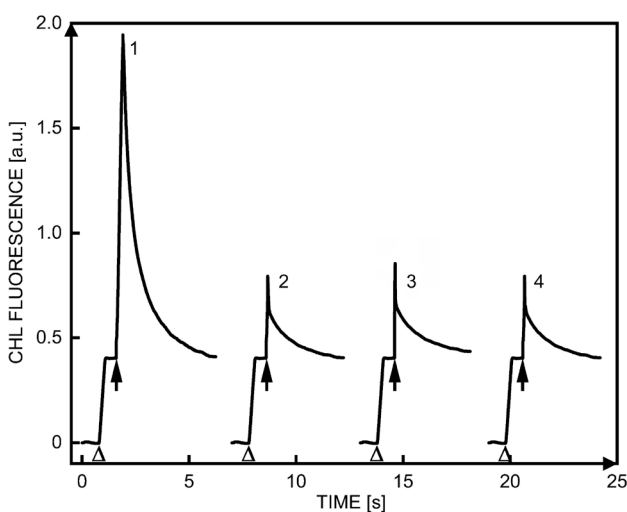


Fig. 2. Kinetics of photoinduced changes in the PSII chlorophyll fluorescence yield measured on spinach thylakoids without additives (kinetics 1), in the presence of $40 \mu M Cu^{2+}$ (kinetics 2), as well as in the presence of $40 \mu M Cu^{2+}$ and $2 mM TOC1$ added simultaneously with Cu^{2+} (kinetics 3) or after incubation of thylakoids with Cu^{2+} (kinetics 4). Chlorophyll concentration is $10 \mu g mL^{-1}$. Triangles mark the moments of switching on the measuring light ($\lambda = 490 nm$; $2 \mu mol(photon) m^{-2} s^{-1}$; xenon flash lamp, 64 Hz; BG39 filter, Schott); upward arrows mark the moments of switching on the short (0.8 s) saturating pulse of actinic light ($\lambda = 490 nm$; $1,200 \mu mol(photon) m^{-2} s^{-1}$; BG39 filter, Schott).

the short saturating pulse (SP) of actinic light [$0.8 s$; $\lambda = 490 nm$; $1,200 \mu mol(photon) m^{-2} s^{-1}$; BG39 filter, Schott] is sufficient to measure the true value of F_m (maximal fluorescence), at which all PSII reaction centers are "closed", since the primary quinone acceptor Q_A is completely reduced to Q_A^- . The full reproducibility of the F_m value in a series of repeated measurements on the same control sample under these conditions [the duration and intensity of the SP are $0.8 s$ and $1,200 \mu mol(photon) m^{-2} s^{-1}$, respectively] showed that irreversible damage to the sample capable of distorting the moment of peak recording does not occur within $0.8 s$.

As shown in Fig. 2, Cu^{2+} ions at a concentration of $40 \mu M$ in the dark decreased the F_m value to $40.9 \pm 1.5\%$ of the control ($p < 0.05$; control taken as 100% ; F_m of control ≈ 1.94 rel. units; in the presence of $40 \mu M Cu^{2+}$ $F_m \approx 0.79$ rel. units; here and hereafter mean $\pm SD$, $n = 5$) (kinetics 2; recording after 5 min of dark incubation). The addition of $2 mM TOC1$ simultaneously with $40 \mu M Cu^{2+}$ followed by dark incubation for 5 min (kinetics 3) caused only a statistically significant but biologically insignificant increase in F_m – to $43.9 \pm 0.9\%$ of the control ($F_m \approx 0.85$ rel. units), *i.e.*, only by ~ 3 percentage points compared to kinetics 2, which does not indicate the elimination of Cu-induced suppression. The addition of $2 mM TOC1$ to thylakoids pre-incubated in the dark with $40 \mu M Cu^{2+}$ for 5 min, followed by incubation as in the first case (kinetics 4), practically did not change F_m : $40.7 \pm 1.8\%$ of the control (in the control, incubation of thylakoids with Cu^{2+} without TOC for 5 and 10 min was not accompanied by additional changes in F_m). The difference between kinetics 2 and 4 is statistically insignificant. Similar results were obtained for TOC2 and TOC3, confirming that their effects are indistinguishable from those of TOC1. Specifically, the simultaneous (direct) addition of $2 mM TOC2$ with $40 \mu M Cu^{2+}$ resulted in an F_m value of $42.5 \pm 1.2\%$ of the control, while its sequential (reverse) addition after Cu^{2+} pre-incubation yielded $41.2 \pm 1.6\%$. For TOC3, the simultaneous addition gave $44.5 \pm 1.4\%$ of the control, and the sequential addition gave $40.3 \pm 1.5\%$. In all cases, the differences compared with the Cu^{2+} -only treatment ($40.9 \pm 1.5\%$) were statistically insignificant. The complete numerical equivalence of the effects observed for all three compounds conclusively demonstrates that neither TOC2 nor TOC3 can prevent or reverse the Cu^{2+} -induced suppression of F_m . Thus, under the described experimental conditions (molar ratio TOC:Cu = 50:1), TOC1–TOC3 did not demonstrate the ability to act as Cu^{2+} ligands. The provided numerical data fully characterize the lack of Cu-chelating activity for the entire series of tested compounds, making the graphical representation of additional kinetic traces redundant for this primary screening stage. Thus, under the described experimental conditions (molar ratio TOC:Cu = 50:1), TOC1–TOC3 did not demonstrate the ability to either prevent (upon simultaneous addition) or eliminate (upon addition after Cu^{2+}) the Cu^{2+} -induced suppression of F_m .

These data are consistent with those available in the literature. On isolated spinach thylakoids, it was shown that $\sim 20 \mu M CuCl_2$ causes 50% inhibition of variable

fluorescence F_v with an unchanged F_0 value, and 40 μM CuCl_2 leads to $\sim 70\%$ inhibition of F_v (Yruela *et al.* 1996). In isolated spinach chloroplasts, at a concentration of 50 μM CuCl_2 , variable fluorescence F_v decreases by $\sim 74\%$ with an unchanged F_0 level (Hsu and Lee 1988). In isolated thylakoids from pea leaves, at a molar ratio of $\text{Cu}^{2+}/\text{Chl} \approx 0.04$ and 0.08 , $\sim 50\%$ and $\sim 90\text{--}100\%$ inhibition of the electron transport rate in PSII was observed, respectively (Mohanty *et al.* 1989). For 50% inhibition of oxygen evolution in PSII membrane fragments, a Cu^{2+} concentration of about $8\text{--}10\text{ }\mu\text{M}$ is required, and in the case of core PSII complexes – about $5\text{ }\mu\text{M}$; with an increase in the Cu^{2+} concentration to $70\text{ }\mu\text{M}$, complete suppression of oxygen-evolving activity occurs (Renger *et al.* 1993). In isolated spinach thylakoid membranes, the F_v/F_m parameter was inhibited by 50% at $35.67\text{ }\mu\text{M}$ Cu^{2+} (PEA) and at $71.00\text{ }\mu\text{M}$ Cu^{2+} (PAM) (Dewez *et al.* 2007). Cu^{2+} causes 50% and complete inhibition of PSII activity in a cyanobacterial mutant at concentrations of 10 and $50\text{ }\mu\text{M}$, respectively (Mishra *et al.* 1993).

Despite some quantitative discrepancies in the absolute values of inhibition percentages obtained at close Cu^{2+} ion concentrations, the general picture fundamentally coincides with the results we obtained. Thus, at $40\text{ }\mu\text{M}$ Cu^{2+} , we recorded a decrease in F_m to $40.9 \pm 1.5\%$ of the control (*i.e.*, inhibition by $\sim 59\%$), whereas in the literature at comparable concentrations ($35\text{--}50\text{ }\mu\text{M}$), the degree of suppression of variable fluorescence or photosynthetic activity of PSII varies in the range from $\sim 50\%$ to $\sim 74\%$. Thus, the order of the effect and the concentration dependence generally agree.

Certain quantitative discrepancies between our data and literature values are quite natural and can be explained by a number of factors. A critical parameter is not the absolute concentration of Cu^{2+} in the solution, but the molar ratio of Cu^{2+} /chlorophyll (or Cu^{2+} /protein) in the sample: in various works, the chlorophyll concentration varied from $20\text{ }\mu\text{M}$ (Renger *et al.* 1993) to $\sim 280\text{ }\mu\text{M}$ (Dewez *et al.* 2007) and $\sim 11\text{ }\mu\text{M}$ (Boucher and Carpentier 1999), which, at the same nominal concentration of Cu^{2+} , leads to a significantly different degree of loading of ions onto the PSII reaction centers. In addition, Cu^{2+} ions actively interact with the components of the buffer solution, which reduces their effective free concentration (Renganathan and Bose 1990), and differences in the incubation medium composition can noticeably change the proportion of Cu^{2+} available for binding to PSII. The method of sample preparation, incubation conditions (temperature, duration, illumination), and the choice of the recorded parameter (F_v , F_v/F_m , O_2 evolution rate, electron transport rate), which have different sensitivities to the same level of damage, have a significant effect (Renger *et al.* 1993). Finally, the nature of the Cu^{2+} salt anion (CuCl_2 vs. CuSO_4) can slightly affect the ionic strength of the solution. The combination of the listed factors explains why, at formally close nominal concentrations of Cu^{2+} , the degree of PSII inhibition in different experimental systems can vary within $10\text{--}20$ percentage points, without contradicting the general pattern observed in our experiments.

As shown in Fig. 1, by themselves (without Cu^{2+} ions), none of the TOCs added to intact thylakoids caused fluorescence quenching. This eliminates the assumption that the TOC itself is a fluorescence quencher. In addition, we ensured that the system we used for primary screening, based on the decrease in the F_m level of chlorophyll fluorescence caused by Cu^{2+} ions and the reversal of this quenching by a presumed Cu^{2+} ion chelator (its prior or subsequent addition to the system), works, which was confirmed by the so-called "positive control": as a reference Cu^{2+} ion chelator, we used EDTA (data not shown). Since the theoretical analysis showed that TOC1–TOC3 differ fundamentally from EDTA/EGTA, the current experimental work was focused strictly on comparing the three TOCs with each other. The reversal of the Cu^{2+} effect using 0.2 mM EDTA (*i.e.*, in a 5-fold excess relative to $40\text{ }\mu\text{M}$ Cu) indicates that under our specific conditions (buffer, thylakoids, equipment), the reversal of the Cu^{2+} effect is possible in principle – EDTA works [as was experimentally shown earlier by the 30% reversal by EDTA of the inhibition of oxygen evolution by Cu^{2+} ions (Pádua *et al.* 2010)], and the new TOC1–TOC3 do not, and the problem lies specifically in the TOCs, and not in a "non-working system". It has been experimentally shown that the inhibition of PSII by Cu^{2+} ions can be removed (reversed) by chelating compounds (EDTA), but only before irreversible structural damage occurs. This is confirmed in several other works (Mishra *et al.* 1993, Arellano *et al.* 1994, Barón *et al.* 1995, Jegerschöld *et al.* 1995). It was shown that the $[\text{Cu}(\text{EDTA})]^{2-}$ complex is inert towards PSII (Kong and Price 2022). $[\text{Cu}(\text{EDTA})]^{2-}$ does not inhibit electron transport in PSII and does not affect fluorescence parameters. The use of $[\text{Cu}(\text{EDTA})]^{2-}$ as a source of copper in nutrient media (like Aquil) completely excludes the toxic effect that would inevitably accompany the introduction of free Cu^{2+} ions, causing oxidative stress and degradation of cellular structures (Kong and Price 2022).

Determining the exact stability constants of complexes and constructing full concentration dependencies is beyond the scope of this brief report.

The lack of pronounced biological activity in the tested TOCs can be structurally explained by the specific spatial arrangement of their pharmacophoric elements. For PSII inhibition, classical herbicides require a precise geometric orientation of hydrogen-bond donors and acceptors to interact with Ser264 and His215 of the D1 protein (Trebst 1986, 1987; Battaglia *et al.* 2021). The fused rigid polycyclic cores of TOC1 and TOC2 lack this specific spatial flexibility, while the thiocarbonyl group ($\text{C}=\text{S}$) in TOC3 differs sterically and electronically from the classical carbonyl ($\text{C}=\text{O}$), altering the hydrogen-bonding topology. Regarding Cu^{2+} chelation, the formation of thermodynamically stable complexes with reference chelators (EDTA/EGTA) relies on a flexible backbone that allows the "wrap-around" formation of multiple 5- or 6-membered chelate rings (Martell and Motekaitis 1992). The rigid, predominantly planar structures of TOC1–TOC3, combined with the absence of polyanionic

carboxylate groups, prevent the formation of such stable 3D coordination cages, resulting in the observed lack of Cu-chelating ability *in vitro*.

Conclusion: Thus, as a result of the primary screening of novel organic compounds as possible inhibitors of PSII and/or ligands of Cu²⁺ ions, it was established that under the described experimental conditions (molar ratio TOC:Cu = 50:1), TOC1–TOC3 did not demonstrate the ability either to change the parameters of PSII chlorophyll fluorescence characteristic of known photosynthesis inhibitors (herbicides) or to reverse the Cu²⁺-induced suppression of F_m, which is inherent to reference Cu²⁺ chelators. This is true for both the direct and reverse order of adding TOCs to the incubation medium at their molar excess.

Theoretical analysis of the structural and functional features of TOC1–TOC3 showed that all three compounds contain separate structural elements potentially relevant both for PSII inhibition and for Cu²⁺ coordination, but none of them fully reproduces the pharmacophoric and physicochemical characteristics of reference compounds (atrazine, diuron, EDTA, EGTA). The experimental data obtained in this work confirmed this prediction: the absence of a urea pharmacophore, high polarity (TOC1, TOC2), low denticity, and the absence of a polyanionic charge (all three TOCs) do not allow expecting pronounced activity within the studied models.

The obtained negative results of the primary screening are themselves of scientific and practical value, since they allow one to: (1) exclude these structural classes from the further search for PSII inhibitors and Cu²⁺ chelators within this study; (2) formulate more stringent structural requirements for candidate molecules of the next generation; (3) verify the proposed combined screening approach as an informative tool for primary evaluation. For a conclusion on the presence or absence of weak/moderate activity, additional targeted studies are necessary: competitive tests with atrazine/diuron, molecular docking into the Q_B site of the D1 protein, spectrophotometric and potentiometric titration of Cu²⁺, EPR spectroscopy, and Job's method for determining the stoichiometry of complexes. It should be noted that while this study focused strictly on the primary screening of PSII photochemistry and Cu²⁺ chelation, the potential off-target effects of TOC1–TOC3 on other photosynthetic pathways or specific enzymatic reactions of the plant cell cannot be entirely ruled out and warrant further targeted investigation.

Since the tested compounds did not exhibit direct PSII inhibitory activity at the screening stage, the question of their reversibility as potential herbicides is currently not applicable. Furthermore, while this study was strictly focused on Cu²⁺ due to its dual role as a micronutrient and toxicant, the potential coordination of TOC1–TOC3 with other transition metals remains an interesting avenue for future research.

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