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Selective *Candida tropicalis* inhibitory bisanthraquinone rugulosin A potentially targets NADPH-cytochrome P450 reductase *in silico*

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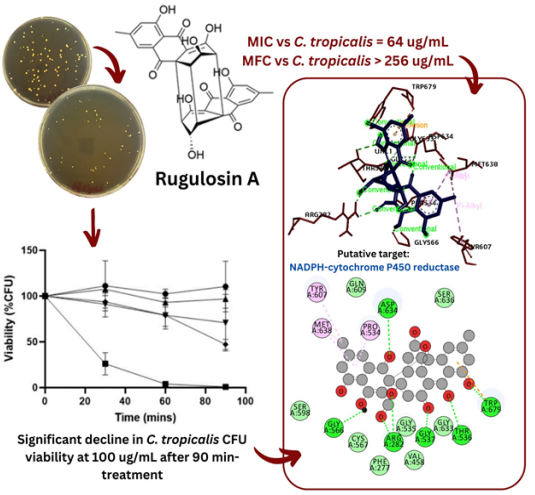
Graphical Abstract



Abstract

The anticandidal activities of the fungal bisanthraquinone metabolite rugulosin A against *Candida albicans* and *Candida tropicalis* were investigated using *in vitro* inhibitory and molecular docking experiments. The colony forming unit (CFU) viability assay used to determine time- and concentration-dependent effects to colony survivability of *C. albicans* and *C. tropicalis* showed that at 100 ug/mL of rugulosin A, the viability of *C. tropicalis* significantly decreased after 90-minute treatments. The minimum inhibitory concentration (MIC) assay, on the other hand, illustrated selective *in vitro* inhibitory activity of rugulosin A against *C. tropicalis* (MIC = 64 ug/mL) over *C. albicans* (MIC > 256 ug/mL). The minimum fungicidal concentration (MFC) assay suggested fungistatic properties for rugulosin A.

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Molecular docking simulations across seven protein targets showed the NADPH-cytochrome P450 reductase, an enzyme important for cell growth of *C. tropicalis*, as the target of rugulosin A being indicated by a strong binding energy of -11.5 kcal/mol in the pocket of its active site.

Keywords: anthraquinone; rugulosin A; molecular docking; *Candida tropicalis*; NADPH-cytochrome P450 reductase

Introduction

Candidiasis or yeast infections caused by *Candida* species are considered global public health concerns due to their increased occurrence and frequency in recent years [1-2]. In the Philippines, candidiasis accounted for approximately 80.40% of fungal infections in 2016 [3-4] - encompassing various types such as recurrent vulvovaginal and oral candidiasis, as well as candidemia. Candidal infections also emerged as the primary reason for consultations at the Dermatology Out-Patient Section of the Department of Health-Research Institute for Tropical Medicine [4]. While *Candida* spp. are common residents of the human microflora, immunocompromised individuals tend to suffer severe systemic infections which may result in morbidity and mortality [5-6]. Candidiasis can be treated using chemotherapeutic agents; however, *Candida* spp. have developed survival mechanisms against these drug-induced pressures rendering current antifungal agents less effective. In addition, there are only four classes of drugs currently available for systemic candidiasis treatment: azoles, polyenes, echinocandins, and pyrimidine [7-9].

Natural products are invaluable starting points for the development of antifungal drugs. Compounds such as polyenes (e.g., amphotericin B) and echinocandins (e.g., caspofungin) from are essential components of the antifungal armamentarium [10]. Numerous natural products with promising anticandidal activities have been identified including phenolics, alkaloids, terpenoids, and peptides - each offering unique mechanisms of action and potential for drug development [11-12].

Among biologically active classes of NPs, anthraquinones (AQs) or quinone-containing compounds have emerged as promising drug templates. While AQs are commonly associated with plants, it is noteworthy that several species of fungi produce these type of bioactive compounds [13-14]. Fungal-derived anthraquinones have captured interest due to their potential in developing novel antifungal agents. Their structural complexity on the other hand offers opportunities for medicinal chemistry optimization. Fungal AQ producers include species from the genera *Penicillium*, *Aspergillus*, *Fusarium*, and *Alternaria*. The production of these secondary metabolites, including anthraquinones, have long been described as a part of fungal defense mechanisms and/or other ecological interactions [15-17]. In the context of anticandidal drug discovery, fungal AQs such as endocrocin, skyrin, and monodictyphenone often exert their antifungal effects by disrupting membrane integrity, inhibiting essential enzymes in candidal infections, or by interfering cellular processes crucial for *Candida* survival [17-18]. As part of our efforts to explore and contribute to antifungal drug discovery based on natural products of plant and fungal origins [19-21], the anticandidal activities of the polyketide-derived bisAQ rugulosin A (Figure 1) against *C. albicans* and *C. tropicalis* using *in vitro* and molecular docking studies are hereby reported.

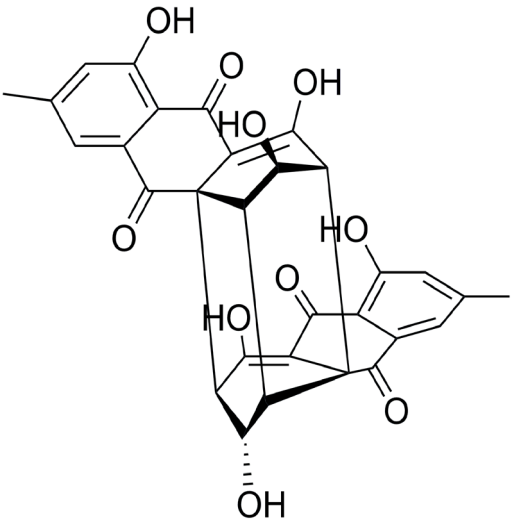

Figure 1. Structure of rugulosin A.

Materials and methods

Test compound. The polyketide-derived anthraquinone rugulosin A was isolated and identified through a combination of chromatographic methods and NMR spectroscopy as previously described by Abanto et al. [22]. Rugulosin A was diluted with dimethyl sulfoxide (DMSO) to achieve the required concentrations (256, 128, 100, 64, 50, 32, 16, 8, 4, 2.5, 2 and 1 µg/mL).

CFU viability assay. Colony forming unit (CFU) viability assay was performed based on methods previously reported by Cascio et al. [23] with slight modifications. Thus, the pathogenic yeast (*C. albicans* ATCC 90028 and *C. tropicalis* ATCC 750) cells were cultured overnight until the exponential phase (OD_{600} = 0.3 to 0.8) was reached. The yeast suspension was serially diluted to attain 700-900 cells per mL followed by addition of rugulosin A (2.5, 50 and 100 µg/mL). From the diluted yeast suspensions, 50 µL was transferred and spread on Sabouraud dextrose agar (SDA) plates after 0, 30, 60, and 90 minutes to measure the time-dependent effects of rugulosin A on yeast CFU viability, and the plates were incubated for 48 hours. DMSO and amphotericin B served as negative and positive controls, respectively. Viability was measured based on the equation below:

$$\% \, CFU = \frac{CFU_{tx}}{CFU_{t0}} \; x \; 100$$

where CFU_{tx} is the CFU count at the specific time point (tx = 30, 60, or 90 minutes) per test concentration and CFU_{t0} is the CFU count at the starting time point.

MIC and MFC determination. The minimum inhibitory concentration (MIC) was determined using the microdilution methods described by CLSI M27-A3 [24]. Briefly, the following concentrations of rugulosin A were prepared via serial dilution in a 96-well microplate: 256, 128, 64, 32, 16, 8, 4, 2 and 1 µg/mL. For the positive control, the following concentrations were used: 8, 4, 1, 0.5, 0.25, and 0.125 µg/mL.

In each well, 100 µL of test concentrations, 5 µL test standardized yeast suspension in Sabouraud dextrose broth (SDB) with 5.0 x 10^2 to 2.5 x 10^3 cells per mL, and 100 µL RPMI 1640 broth medium were combined and incubated (48 hours). Absorbance values were read at 600 nm using Promega Glomax Microplate Reader. Normalized absorbance values were computed by subtracting the absorbance readouts to the readout values for blank (same solution but without yeast inoculum). The vehicle control DMSO and anticandidal agent amphotericin B served as negative and positive controls, respectively. The concentration that induced significant decrease in OD_{600} was considered the MIC.

Minimum fungicidal concentration (MFC) was determined using the same set of protocols in CLSI M27-A3 [14]. 10 µL solution from the well containing the MIC, and two concentrations higher, was inoculated and spread on SDA plates. The concentration that promoted no colony growth after 48 hour-incubation of plates was considered as the MFC. DMSO and amphotericin B served as negative and positive controls, respectively.

Molecular docking experiments

Protein and ligand preparation. The following PDB IDs served as the protein targets: 6T1U (NADPH-cytochrome P450 reductase or CYPOR), 1EQP (1,3-β-glucan synthase), 4QUV (δ-14-sterol reductase), 5TZ1 (lanosterol 14-alpha demethylase or CYP51), 5UIV (thymidylate kinase), 4LEB (Als3 adhesin), and 2Y7L (Als9-2). These seven proteins were processed through the removal of co-crystallized structures and non-standard residues and minimized using default parameters in the UCSF Chimera (1.17.3) [25]. The PDB IDs used have already served as key targets in other molecular docking studies. In addition, the receptor choice was determined based on the current medication targets advised for managing candidiasis [26-29]. Most studies demonstrated that pharmaceutical targets in *C. albicans* may apply to other pathogenic *Candida* spp. [30]. Rugulosin A was prepared in Avogadro (1.2.0) for optimization after inputting SMILES notation in the UCSF Chimera software.

Molecular docking simulations and interaction visualization. Both protein targets and the ligand rugulosin A in PDB and mol2 formats, respectively, were fed in UCSF Chimera (1.17.3) for actual molecular docking simulations using the flexible ligand into a flexible active site protocol. The grids that encompass the target binding domains were generated (Table 1). A total of 10 binding modes were analyzed using the BFGS algorithm in AutoDock Vina. Interaction visualization was performed in BIOVIA Discovery Studio Visualizer (4.1) [31].

Table 1. Parameters utilized for the generation of grids to encompass the target binding domains.

Protein Targets		Coordinates			Size (Å)		
PDB IDs	**Protein Names**	***x***	***y***	***z***	***x***	***y***	***z***
6T1U	cytochrome P450 reductase	-16.15	-5.75	-51.25	44.93	34.06	35.89
1EQP	exo-β-(1,3)-glucanase	33.30	38.81	54.19	58.58	41.97	46.74
4QUV	δ-14-sterol reductase	-20.124	-3.19	27.40	28.51	36.62	34.86
5TZ1	lanosterol 14-alpha demethylase (CYP51)	69.82	68.77	5.86	32.23	32.14	33.17
5UIV	thymidylate kinase	31.74	18.52	13.15	23.99	27.35	23.23
4LEB	Als3 adhesin	8.17	2.29	-19.25	26.63	22.83	25.95
2Y7L	N-terminal domain of Als9-2	-2.34	24.66	-14.94	39.80	27.88	50.52

Table 2. MIC and MFC of rugulosin A against *C. albicans* and *C. tropicalis*.

Test compounds	MIC (ug/mL)		MFC (ug/mL)	
	C. albicans	***C. tropicalis***	***C. albicans***	***C. tropicalis***
Rugulosin A	>256	64	-	>256
Amphotericin B	0.5	0.5	0.833	1.67
DMSO	>256	>256	-	-

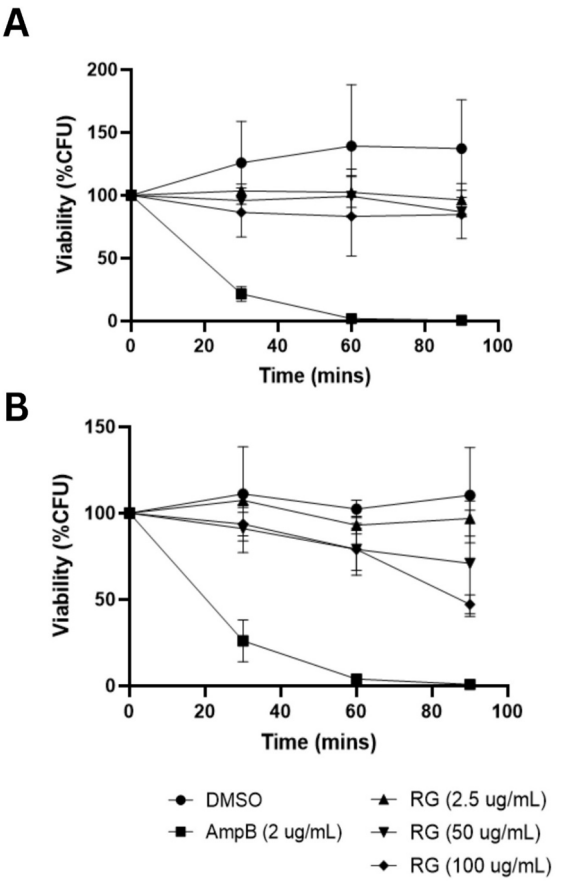

Figure 2. CFU viability of (a) *C. albicans* and (b) *C. tropicalis* after exposure to rugulosin A for 0, 30, 60 and 90 mins.

Results

The antifungal activities of rugulosin A were investigated for inhibitory and/or fungicidal effects against the pathogenic yeast species, *Candida albicans* and *C. tropicalis*. All concentrations of rugulosin A did not induce significant reduction in colony forming unit (CFU) viability of *C. albicans* during the 90-min exposure time. Meanwhile, at 100 ug/mL test concentration, rugulosin A promoted significant decline in the percentage of viable *C. tropicalis* CFUs 90 min post-treatment (Figure 2).

To validate the *in vitro* anticandidal activities of rugulosin A, the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) vs *C. tropicalis* and *C. albicans* were determined. The lowest concentration that significantly decreased OD_{600} of the non-treated inoculum in the microwells was considered as MIC while MFC is the lowest concentration that completely inhibited the growth of yeast colonies [24]. The MIC value of rugulosin A indicated that susceptibility was only observed in *C. tropicalis* (Table 2). This result corroborated with the CFU viability assay results where *C. albicans* conferred resistance to the anthraquinone. As for the results of MFC determination, a much higher concentration (> 256 ug/mL) was needed to elicit fungicidal effects of rugulosin A. Thus, the anti-*C. tropicalis* activity may be limited to fungistatic or growth-inhibitory mechanisms.

While rugulosin A exhibited potential selective fungistatic activity vs *C. tropicalis*, further investigations necessitates the exploration of its potential candidal protein targets. Molecular docking simulations were conducted using emerging targets *in silico* for anticandidal drug discovery studies. Thus, rugulosin A showed best binding affinity to 6T1U (NADPH-CYPOR) with BE of -11.5 kcal/mol over the other six protein targets (Table 3). The rugulosin A/6T1U complex established the highest number of H-bonds which might have contributed to the highly favorable binding energy. The H-bond-forming residues include Trp679, Asp634, Gly537, Thr536, Arg282, and Gly566. The other residues noted for their intermolecular interactions with rugulosin A were Gly633, Gly535 (C-H bond), Trp679 (*pi*-anion), Met638, Pro534, Tyr607 (alkyl / *pi*-alkyl) (Table 3; Figure 3). Compared to the positive controls, rugulosin A demonstrated better binding activity across all protein targets.

Discussion

A number of anthraquinones (AQs) such as rugulosin A has been found to target cancer cell DNA [32-33], affect autophagy and hypoxia, induce apoptosis and paraptosis, and inhibit metastasis and invasion. However, only a limited number has been explored for anticandidal properties, with mechanisms of action being poorly understood. For example, rugulosin A and skyrin were shown to inhibit certain microbial pathogens but not *Pseudomonas aeruginosa* and *C. albicans*. In addition, (+)-2,2'-epicytoskyrin A demonstrated strong inhibition against *Candida* spp., including *C. albicans* and *C. tropicalis* [34]. The study therefore contributes to the growing interest in tapping AQs for anticandidal drug discovery.

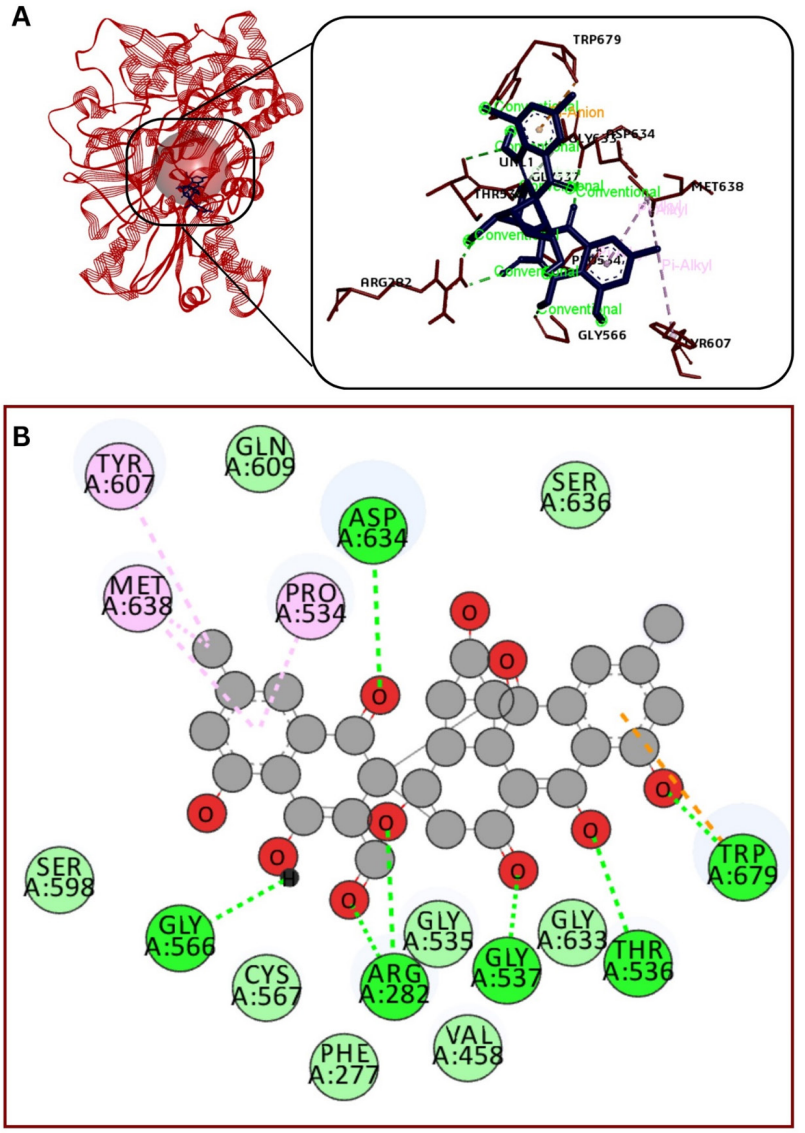

Figure 3. (**a**) Dock pose of rugulosin A and *C. tropicalis* NADPH-cytochrome P450 reductase complex and (**b**) the binding interactions and involved residues.

Table 3. Binding affinity and interactions between rugulosin A and target candidal proteins.

PDB IDs	Protein Names	Rugulosin A		Positive Controls BE (kcal/mol)		
		BE (kcal/ mol)	Interactions	Caspofungin	Amphotericin B	Co-crystallized ligand / inhibitor
6T1U	cytochrome P450 reductase*	-11.5	Trp679, Asp634, Gly537, Thr536, Arg282, Gly566 (H-bond), Gly633, Gly535 (C-H bond), Trp679 (*pi*-anion), Met638, Pro534, Tyr607 (alkyl / *pi*-alkyl)	-8.8	-9.2	-
1EQP	exo-β-(1,3)-glucanase	-10.6	Asp145, Tyr153 (H-bond), Phe144, Leu194, Pro196 (alkyl / *pi*-alkyl), Phe144 (*pi*-*pi* stacked)	-8.0	-8.8	-
4QUV	δ-14-sterol reductase	-8.5	Tyr407, Arg323, Lys259, Tyr360 (H-bond), Asp399 (C-H bond), Thr254 (amide-*pi* stacked), Met99 (alkyl)	-7.2	-7.5	-9.5
5TZ1	lanosterol 14-alpha demethylase (CYP51)	-10.0	Leu376 (*pi*-sigma), Gly307 (amide-*pi* stacked), Ile379, Ala476, Cys470 (alkyl / *pi*-alkyl)	-5.7	-3.3	-10.6
5UIV	thymidylate kinase	-9.2	Arg92, Arg39, Ser18 (H-bond), Gly155, Asp13 (C-H bond), Arg39 (unfavorable donor-donor), Glu159, Arg92, Arg39 (*pi*-cation / *pi*-anion), Ty161 (*pi*-alkyl)	-8.2	-7.7	-8.9
4LEB	Als3 adhesin	-10.4	Thr20, Asn22, Thr168, Tyr23 (H-bond), Val161 (*pi*-sigma), Leu293, Tyr166 (alkyl / *pi*-akyl)	-6.5	-7.7	-
2Y7L	*N*-terminal domain of Als9-2	-8.5	Thr168 (H-bond), Tyr21, Val161, Leu292 (*pi*-alkyl	-6.4	-7.1	-

(-) not applicable / not determined; *the only PDB structure from *C. tropicalis*; the rest were from *C. albicans* which can be applied to non-*albicans* yeast pathogens [30].

To date, limited studies disclosing the selective effects of rugulosin A against *C. tropicalis* over *C. albicans* are reported. The sensitivity and selectivity in anti-candidal activity highlights an important concept in antifungal drug discovery especially that non-*albicans* species continue to emerge in terms of virulence and epidemiology. *C. tropicalis* is known to exhibit strong adherence to host epithelial and endothelial cells and is an osmotolerant microbe. These features are attributed to increased survival in highly saline environments and antifungal resistance mechanisms [35-36]. *C. tropicalis* is the most prevalent yeast species detected among candidemia patients [37].

Most anticandidal studies on AQs have been so far focused on *in vitro* screening through evaluation of MIC and MFC, emphasizing lack of thorough investigations and/or predictions in understanding their mechanisms of action. Using molecular docking, we identified the potential target of rugulosin A as NADPH-cytochrome P450 reductase (CYPOR), which may explain its ability to inhibit the growth of *C. tropicalis*. This enzyme is crucial for activating and deactivating different cellular compounds such as hormones, fatty acids, and sterols. NADPH-CYPOR is considered a vital part of the cytochrome P450 enzyme system responsible for converting lanosterol to ergosterol, a critical component of fungal membranes. Blocking this enzyme system can disrupt sterol production, leading to membrane instability and reduced cell growth [38-40]. Moreover, the enzyme plays a role in detoxifying harmful substances and antifungal agents by transforming them into less harmful forms, which is essential for candidal survival in challenging environments [41-42].

While rugulosin A demonstrated the highest binding propensity to NADPH-CYPOR based on binding energy, the dock complex also recorded the highest number of hydrogen bonds formed. Hydrogen bonds can significantly contribute to the binding affinity between a ligand and its protein target. Strong and well-oriented hydrogen bonds between complementary functional groups can stabilize the ligand-protein complex, leading to higher binding affinities. Molecular docking algorithms often prioritize ligand poses that form optimal hydrogen bond interactions with the protein, as these interactions contribute to the overall stability of the complex [43-47]. Other than the formation of H-bonds, the highest number of other interactions e.g., C-H bond, *pi*-anion, *pi*-alkyl, and alkyl was also noted. These interactions may have contributed to the relatively favorable binding between the ligand and NADPH-CYPOR and further strengthened the binding activity [46-47].

Conclusion

The present study reports selective inhibition of the bisanthraquinone rugulosin A against *Candida tropicalis in vitro*. The NADPH-cytochrome P450 reductase as the putative molecular target of rugulosin A in *C. tropicalis* cells was also predicted. To propel studies on rugulosin A as a pharmacophore and/or drug template in discovering new anticandidal agents, further investigations are warranted.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization, methodology, data collection, data analysis and interpretation, original draft preparation, review and editing of the draft, J.A.H.M. Conceptualization, data analysis and interpretation, review and editing of the draft, supervision, A.P.G.M., N.P.G.A. All authors have read and agreed to the final version of the manuscript.

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Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

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