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Synthesis of New Coumarin Derivatives Used as Nitrification Inhibitors to Mitigation of Nitrous Oxide Emission from Agricultural Soil.

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Abstract:
Nitrous oxide (N_2O) is one of the major greenhouse gases "GHG" the main source of N2O emission is synthetic mineral nitrogen fertilizers. The conversion values according to the global warming potential for N_2O are 298 times as CO_2 per molecule. The main objective of this work is to mitigate GHG emission from N_2O by create synthesis new nitrification inhibitors. Coumarine derivatives were synthesized **(1-4)**, and prove the structures by spectral data, the compounds (**1**) and (**4**) were applied as nitrification inhibitors to mitigation of nitrous oxide. The results indicated that, the presence of each of the two tested inhibitors (**1**, **4**) reduced the N_2O emission from urea-treated soils by 72% and 49%, respectively, in inhibiting nitrification during the 28-day incubation period.
Key words: Nitrous oxide emissions, Mitigation, Agricultural soil, Nitrification, Coumarin derivatives.

1. *Introduction*:

The atmospheric N_2O concentration before the industrial revolution was 0.28–0.29 ppm (1) and it currently reached to 0.31 ppm (2, 3). Nearly two-thirds of agricultural emissions occur as N_2O, which has 298 times the global warming potential of CO_2 (4) .The N_2O is considered responsible for 4 to 5% of global warming (5, 6), being the tropical and temperate noncultivated soils the main sources and accounting for 63% of N_2O total emissions (6). Several ideas for N_2O mitigation have been suggested (7), which include optimum N supply to crops, proper animal and crop residue management, use of controlled-release fertilizers, nitrification inhibitors, and proper water management, etc. Nitrification inhibitors can delay the microbial oxidation of NH_4^+ to nitrite (NO_2^-) for a certain period (several weeks or months) and are therefore very effective in blocking microbial nitrification and subsequent denitrification (8,9). The effects of more than 100 compounds (organic and inorganic) on soil unease activity were studied (10). At commercial level, it is possible to find nitrification inhibitors such as nitrapyrin (2-chloro-6- trichloromethylpyridine; NP), dicyandiamide (2-cyanoguanidine; DCD) and 3,4-dimethylpyrazole phosphate (DMPP) (11,12), with diverse results. Many plant products, especially non-edible oilseeds and their constituents have been found to inhibit nitrification in soil (13). Among them, karanjin (3-methoxy furano-2′,3′,7,8-flavone), a furano-flavonoid, which obtained from karanja (*Pongamia glabra* Vent.) seeds, and have excellent nitrification-inhibiting activity (14,15). Different alternative forms of karanjin such as karanj-ketone (4-hydroxy- 5–methoxy acetyl coumarine) inhibit nitrification. Other modifications, where the coumarin ring is kept intact, retain this effect in varying degrees. The main target of this work to diagnose the nitrification inhibition efficiency of tow synthetic coumarin derivatives in urea –fertilized soils in order to investigate their profitable role in reducing N_2O emissions.

Materials and Methods:

2.1. Soil

The surface soil (0–15 cm) was collected from the research farm of Central Laboratory for Agricultural Climate (CLAC), Agricultural Research Center (ARC),Dokky ,Egypt. The soil analysis are recorded in Table (**1**)
The soil moisture content at field capacity 25%.

Table (1) Some physical and chemical characteristics of the investigated soil.

Soil parameter	Value
pH	7.4
EC(mmoh/cm)	5.2
	Soluble cations (m molc L-1)
Ca^{++}	22.4
Mg^{++}	33.4
Na^{+} mmoh/l	20
K^{+} mmoh/l	7.5
	Soluble anions (mmolcL-1)
Cl^{-}	41
CO_3^{-}	1.1
HCO_3^{-}	13
SO_4^{--}	28.2

2.2. Preparation of inhibitors:

To synthesis the two tested nitrification inhibitors (**1**, **4**) , the following reagents were prepared in the series steps:

2.2.1. Synthesis of (7-hydroxy-2-oxo-2H-chromen-4-yl) acetic acid (1):

The (7-hydroxy-2-oxo-2H-chromen-4-yl) acetic acid prepared as a procedures reported (16).

2.2.2. Synthesis of 2-(7-hydroxy-2-oxo-2H-chromen-4-yl) acetyl chloride (2):

The product (**2**) was prepared as reported (17).

2.2.3. Synthesis of 2-(1-(7-hydroxy-2-oxo-2H-chromen-4-yl)vinyloxyamino)- 3-(4-hydroxyphenyl)propanoic acid(3):

A **s**olution of acid chloride **(2; 0.01 mole)** in acetone (30 ml) was added to L Tyrosine **(0.011 mole)** in ethyl alcohol (30 ml) and base, the reaction mixture was stirred for two hours. After the solvent removed under reduced pressure, the crude product of compound **(3)** was obtained. The solid product was filtered and recrystallized from benzene to give (**3**) as brownish red crystals, Yield: (85 %); R_f=0.87; M.P :90-92°C. IR: υ/ cm^{-1} = 3469(OH), 3204(NH), 1720(C=O acid), 1664(C=O ketone), 1594(C=O Amide); ^{H}NMR(DMSO) δ/ppm = 7.35,7.25,7.10 (3H,aromatic protons), 6.25,6.39,6.48 (t,3H,coumarin), 8.8(s,1H,OH)

2.2.4.Synthesis of methyl 2-(2-(7-hydroxy-2-oxo-2H-chromen-4-yl) acetamido)-3-(4-hydroxyphenyl) propanoate (4):

The pure thionyl chloride (0.01 mole) was added in drops with acid (**3**) in absolute methanol (30 ml.) during 30 mins with stirring. The stirring was continued for additional 3 hrs. The mixture was left for 24 hrs at room temperature. The residual material recrystallized by benzene to give product (**4**) as brown crystals. Yield: (87 %); R_f =0.67; M.P:155-57°C, IR υ/ cm^{-1} = 3694(OH), 3435(NH), 1724(C=O), 1598(Amide C=O); ^{H}NMR (DMSO) δ/ppm = 9.31(s, 1H, OH), (5.9-7.4) (m, 8H, aromatic protons).

2.3. Stepwise Docking Method:

2.3.1 Preparation of Small Molecule:

Molecular modeling of the target compounds were built using MOE, and were minimized their energy with PM3 through MOPAC. Our compounds were introduced into the (1KAU) binding sites according to preduction binding stie of the program.

2.3.2. MOE Stepwise

The crystal structures of the (1KAU) with (1) as an reference inhibitor molecule was used, Water and inhibitor molecule was removed, and hydrogen atoms were added. The parameters and charges were assigned with MMFF94x force field. After alpha-site spheres were generated using the site finder module of MOE. The optimized 3D structures of molecules were subjected to generate different poses of ligands using triangular matcher placement method, which generating poses by aligning ligand triplets of atoms on triplets of alpha spheres represented in the receptor site points, a random triplet of alpha sphere centers is used to determine the pose during each iteration. The pose generated was rescored using London dG scoring function. The poses generated were refined with MMFF94x forcefield, also, the solvation effects were treated. The Born solvation model (GB/VI) was used to calculate the final energy, and the finally assigned poses were assigned a score based on the free energy in kJ/mol

2.3. Treatments:

One hundred grams of loamy soil in glass beakers (250 ml) was treated with an 100 ppm urea. Each of tow investigated compound P1and P4 was added to the soil at the rates of 100, 200 and 300 ppm . Solutions, prepared by dissolving crystals of compounds in acetone. Acetone and compounds in acetone were added drop by drop to the soil at room temperature and the beakers were constantly stirred to ensure the immediate evaporation of added acetone. The soil was again thoroughly stirred subsequently, to ensure uniform mixing of the fertilizer N and the inhibitors. Treatments containing control without any inhibitors were also included, thus making 28 treatments in all. The moisture loss from the beakers during the experiment was measured by using soil moisture meter (PMS-714). The gas samples were immediately analyzed for N_2O using Gas filter correlation N_2O analyzer, Model 46i which detect N_2O gas sample by using IR source in the analyzer. These analyzer one of three analyzers for detection Greenhouse Gases (N_2O, CO_2 and CH_4) in a Greenhouse Gases Monitoring Station at Central Laboratory for Agricultural Climate (CLAC), Giza, Cairo.

2.4. Statistical Analyses:

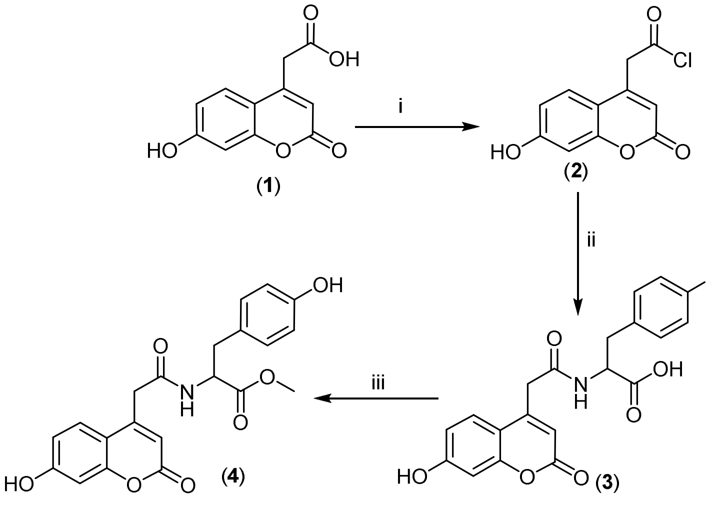
The experimental data was subjected to statistical analysis of variance according to **Snedecor and Cochran (1980)** (18). Duncan test was used to verify means. Data were statistically analyzed using the general linear model procedure of SAS (SAS, 2002,V9) (18). Simple linear regression analyses were carried out to find out functional relationships between the total N2O emission and different concentrations of inhibitor applied.

3. Results and Discussion:

3.1. Chemistry:

The synthetic route employed to produce the series of compounds are portrayed in (Scheme .1).

Acid chloride (2) was prepared by reaction of phosphorous pentachloride with acid (1), the compound (2) was coupled with L-tyrosine in presence absolute ethanol to give compound (3). The IR spectrum of compound (3) was indicated that, the presence of a OH and NH function at 3469, 3204 cm^{-1}, and it's the ^{1}HNMR spectrum showed, a singlet at (δH 8.8 ppm) due to OH protons of carboxylic acid. Methylation of free amino acid derivative (3) afforded corresponding ester (4). Disappearance characteristic OH group from HNMR and IR spectra which confirmed the structure.



Scheme.1:
regant and condition:i: $SOCl_2$,ii: L Tyr/EtOH ,iii $CH_3OH/SOCl_2$

3.2. N_2O Emissions:

The results obtained in this experiment s with compound (**1**) and compound (**4**) at 0,100,200 and 300 (ppm) concentrations are given in Table (**2**). Their various effects are discussed below:

Table (2) Effect of new inhibitors on N_2O emissions in incubated soil after 7, 15, 22 and 28 days

Inhibitors		N2O Emissions (ppm)				Mean	Mitigation %
Name	**Conc.(ppm)**	7day	15day	22day	28day		
Un treated	**0**	0.67^{c}	0.83^{b}	0.5^{c}	1.0^{b}	0.8	_
Urea (Control)	**100**	25^{bc}	80^{a}	82.0^{a}	105.0^{a}	73.0	0
Comp(1)	**100**	66.3^{ba}	40.6^{ba}	2.8^{c}	1.3^{b}	27.8	72
	200	36.7^{bc}	27.6^{ba}	24.2^{bc}	0.9^{b}	22.4	
	300	40.7^{bc}	6.5^{b}	2.5^{c}	0.5^{b}	12.6	
Comp(4)	**100**	98.0^{a}	43.0^{ba}	49.3^{bc}	1.8^{b}	48.0	49
	200	72.7^{ba}	48.3^{ba}	6.9^{c}	0.8^{b}	32.2	
	300	61.3^{ba}	44.0^{ba}	17.3^{bc}	0.7^{b}	30.8	
LSD		49.4	58.3	40.7	1.7		
Product (P1)		47.9	24.9	9.9	0.9	20.9	72
Product (P4)		77.3	45.1	24.5	1.1	37.0	49
Conc. Mean	**Un treated**	0.67	0.83	0.5	1.0	0.8	_
	control	25.0	80.0	82.0	105.0	73.0	0
	100	82.2	41.8	26.1	1.5	37.9	47
	200	54.7	38.0	15.6	0.9	27.3	62
	300	51.0	25.3	9.9	0.6	21.7	70
General Mean		62.6	35.0	17.2	1.0	29.0	60

Least Significant Difference (LSD): Means with different superscripts between the emissions of N_2O by using two compounds (**1**) and (**4**) (a, b, c, ba, bc) and with urea differ significantly .

Table (**2**) demonstrated that, all the applied nitrification inhibitors treatments are effective and in all cases the effect was increased with increasing concentration. Generally it is evident that, the performance of free acid (**1**) was better than amino acid methyl ester (**4**) at all concentrations, for increasing the percentage of mitigation (72 *V.S.* 49%, respectively). These compounds (**1** and **4**) were tested at 100 ppm urea concentration. Maximum accumulation of N_2O for (**1** and **4**) with different concentration treatments was observed, after first week of incubation showed comparable results with urea. The regulatory effect lasted with all compounds (**1** and **4**) up to 28 days. However compounds (**1**) and (**4**) remained effective beyond this period even at 300, 200 and 100 (ppm) respectively. The effect of mitigation of N_2O emission for compound (**1**) and compound (**4**) are given in figures **1** and **2**.

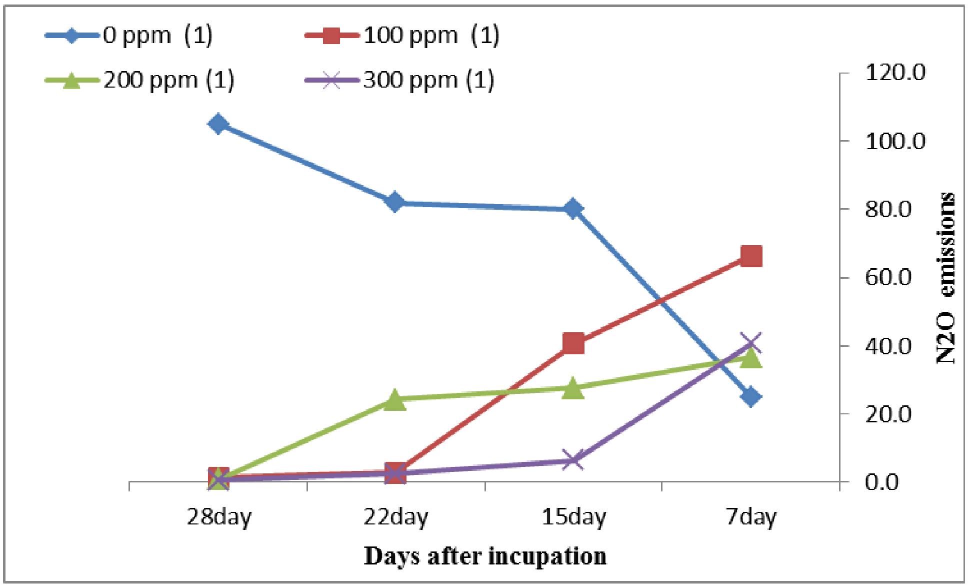


Fig (1) Effect of compound (**1**) on production of N_2O emissions in incubated soils.

In this respect, soil incorporated with **1&4** compounds at 300 ppm for each sharply reduced N_2O emissions after 28 days from incubation by 99.5% and 99.3%, respectively. The potency of these treatments are striking and asserting the decisive need for such inhibitors reagent to mitigate N_2O emissions from urea –fertilized cultivated area.

Data in Table (2) cleared that N2O emissions from solely urea- treated soil were progressively increased as time of incubation was increased. N_2O emissions at 28 day was more 4 fold than that flow out after 7day, the reverse is true with the application of new nitrification inhibitors, irrespective to its concentration. N_2O emissions was gradually degradated and mitigated down, to be less than 0.01%-0.12%of its emission concentration base after 7days from incubation .Our results are comparable to the nitrification inhibition by karanjin compound which reported by earlier(14,19).

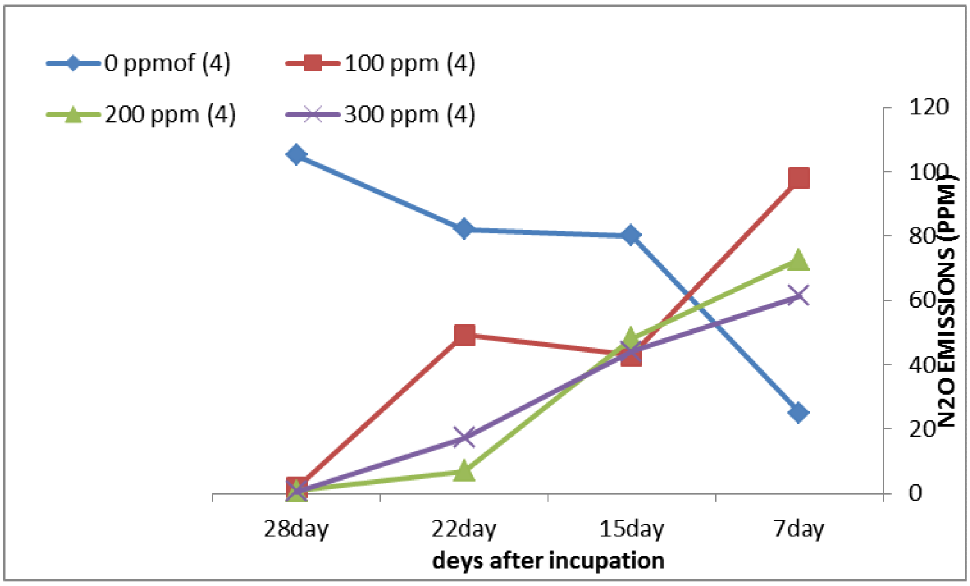
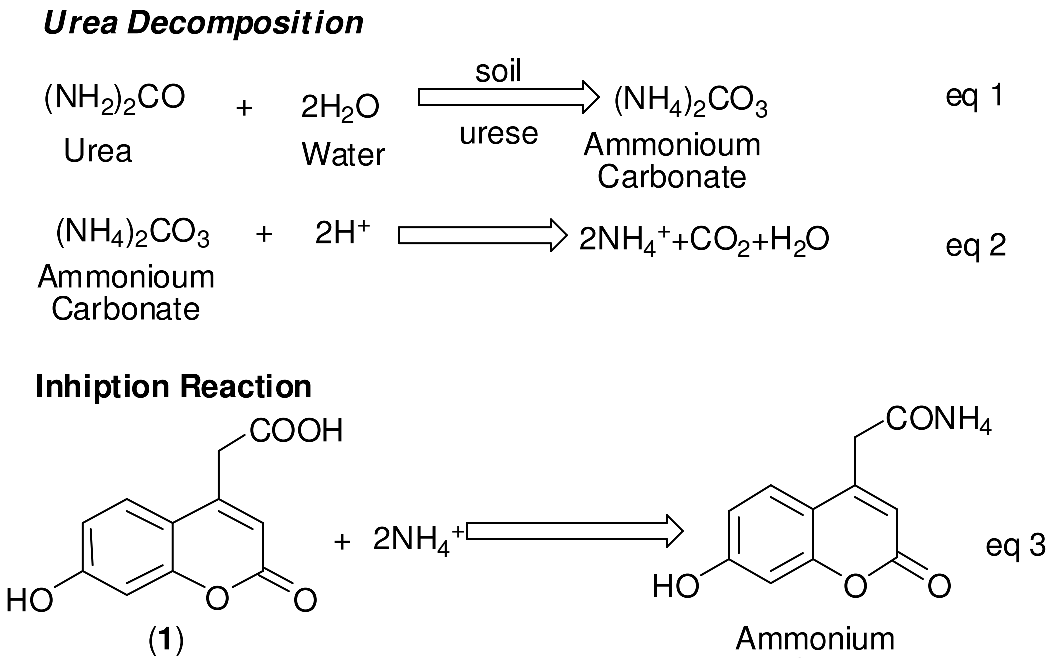


Fig (2) Effect of compound (**4**) on production of N_2O emissions in incubated soils.

It is important to note that, the four tested compound (**1**) and compound (**4**) were showed the highest nitrification inhibitor activity, The present studies further reveal that the carboxylic group in compound (**1**) is essential for this activity, which carboxylic group was reacted with NH_4 in soil to give ammonium salt compound as described in (Scheme.2).



Scheme .2

3.2. *Docking studies:*

All calculations for docking experiment were performed using MOE 2008.10 [20]. The tested compounds were evaluated in silico (docking), using X-ray crystal structures (ID: 1KAU; [21]). The tested compounds were docked into active sites predicted by MOE. The active site of the enzyme was defined, to include residues within a 10.0 Å radius to any of the inhibitor atoms. The scoring functions were applied for the most stable docking model to evaluate the binding affinities of the inhibitors, which complexes with (1KAU) active site. The complexes were energy-minimized with an MMFF94 force field [22] until the gradient convergence 0.05 kcal/mol was reached. All tested compounds (**1**) and **(4)** were docked successfully into the active sites. The compounds **(1)** has higher binding scores compared with **(2)** with values (-154.265 and-112.369) Kcal/mol, (Figures. 3 and 4).

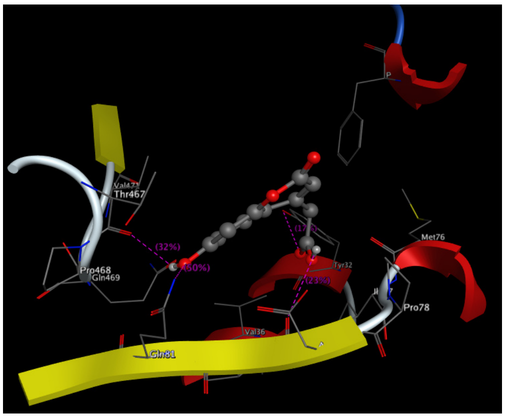


Figure 3. Model of the active site of native KAU, showing the position of selected relevant residues. The C atoms shown in grey, in white, N atoms in blue, and O atoms in red. H-bonds are shown as dashed red lines. All residues are sized according to their different depths.

Figure3 and 4 showed that, Compound 1 was interacted with binding site by formation of one hydrogen bond with Tyr-385 in MVD tool, its compound 12 was stabilized in MOE by formation three H-bond interaction, while compound (4) form weak bond with binding site. The higher binding scores and strong interaction with binding site for compound **(1) compared (2),** explained the higher potency of compound **(1)**.

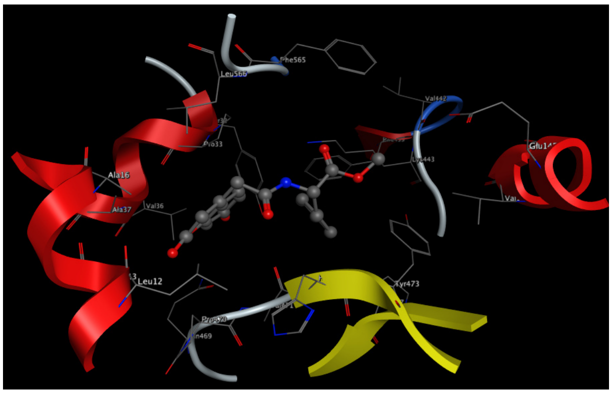


Figure 4. Model of the active site of native KAU, showing the position of selected relevant residues. The C atoms atoms are shown in grey, in white, N atoms in blue, and O atoms in red. All residues are sized according to their different depths.

4. Conclusion

All the nitrification inhibitors used in this study were effective in reducing N_2O emission (Table. **2**). Compound (**1**) and (**4**) with urea were found, most effective in reducing N_2O emission by 72% and 49%, respectively. Lower emission with inhibitors was due to availability of less amount of nitrate for denitrification due to the inhibition of the nitrification process.

A suitable method for the application these inhibitors with fertilizers has to be formulated for its optimum effect under field conditions, as it may not be feasible to apply organic chemical compound mixed in acetone to crop fields at a large scale. Thus, a comparative field evaluation of the efficacy of possible modes of application of 7- hydroxyl coumarin - 4- acetic acid and its derivatives (i.e. inhibitor mixed with fertilizer; inhibitor coating on fertilizer granules; fertilizer coated with inhibitor powder; or inhibitor powder applied directly to soil with fertilizers) would be appropriate. Moreover, the soil residual effect of the promising new product of germination and establishment of field crops must be considered. The molecular docking experiment preformed and show that, The higher binding scores and strong interaction with binding site for compound **(1) compared (2),** explained the higher potency of compound **(1)**.

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