In Situ Acetaldehyde Synthesis for Carboligation Reactions

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The enzyme 4-oxalocrotonate tautomerase (4-OT) can promiscuously catalyze various carboligation reactions using acetaldehyde as a nucleophile. However, the highly reactive nature of acetaldehyde requires intricate handling, which can impede its usage in practical synthesis. Therefore, we investigated three enzymatic routes to synthesize acetaldehyde in situ in one-pot cascade reactions with 4-OT. Two routes afforded practical acetaldehyde concentrations, using an environmental pollutant, *trans*-3-chloroacrylic acid, or a bio-renewable, ethanol, as starting substrate. These routes can be combined with 4-OT catalyzed Michael-type additions and aldol condensations in one pot. This modular systems biocatalysis methodology provides a stepping stone towards the development of larger artificial metabolic networks for the practical synthesis of important chemical synthons.

Introduction

The enzyme 4-oxalocrotonate tautomerase (4-OT) naturally catalyzes the tautomerization of 2-hydroxyhexa-2,4-dienedioate to 2-oxohex-3-enedioate using the N-terminal proline as key catalytic base.[1] In addition, 4-OT can promiscuously catalyze several carbon–carbon bond-forming reactions, including Michael-type additions and aldol condensations, employing Pro-1 as a nucleophile.[2–4] Most notably, 4-OT can catalyze Michael-type additions and aldol condensations using the highly reactive acetaldehyde (**5**) as a nucleophile (Scheme 1).

However, the use of **5** in enzymatic reactions causes several practical challenges. Compound **5** is toxic, highly volatile and reactive, which requires intricate handling. Hence, in situ generation of **5** from less reactive and less toxic compounds is an attractive concept to combine with 4-OT in one-pot cascade reactions. Previous studies have primarily focused on in situ generation of longer aldehydes, for instance from their corresponding carboxylic acids or alcohols using carboxylic acid reductases (CAR) or alcohol dehydrogenases, respectively.[5–8] Here we investigate three enzymatic routes for the in situ generation of **5** in one-pot cascade reactions with 4-OT, using **1**, **3** or **4** as starting substrates (Scheme 1). Route I involves the dehalogenation of **1** into **2**, catalyzed by the enzyme chloroacrylic acid dehalogenase (CaaD), followed by the decarboxylation of **2** into **5** by the enzyme malonate semialdehyde decarboxylase (MSAD). Route II involves the oxidation of **3** into **5** catalyzed by the alcohol dehydrogenase from *Saccharomyces cerevisiae* (*Sc*ADH), in combination with a commercially available NADH oxidase, PRO-NOX(009), to shift the unfavorable reaction equilibrium and to recycle the co-factor. Route III involves the decarboxylation of **4** using the pyruvate decarboxylase from *Zymomonas mobilis* (*Zm*PDC).

Routes I and II proved to be effective routes for in situ generation of **5** and could be used in combination with 4-OT to synthesize **7** and **9** in one pot. Interestingly, route II afforded a mixture of **9** and **10**. The presented work provides a stepping stone towards the construction of artificial enzymatic metabolic routes for the synthesis of valuable chemical commodities.

Results

There are several enzymes that naturally produce **5** as their main product, as **5** is at the crossway of several metabolic routes. We selected three complementary enzymatic routes (I, II and III, Scheme 1) for in situ generation of **5**, using the readily accessible starting materials **1**, **3** and **4**. We initially focused on the enzymatic synthesis of **5** via route I. The release of HCl and carbon dioxide in the first and second step, respectively, is expected to shift the equilibrium towards the formation of **5**. Indeed, initial experiments showed that a cascade reaction with CaaD and MSAD resulted in near quantitative conversion of **1** into **5** (Figure 1). Notably, no significant enzyme inhibition occurred at this concentration of **5**. Optimizing enzyme concentrations afforded 50 mM **5** in 90 minutes using 5 μM CaaD and 1 μM MSAD (Figure 1).

We next focused on the enzymatic oxidation of **3** (Scheme 1, route II), which can be catalyzed by both alcohol oxidases and alcohol dehydrogenases. As alcohol oxidases are prone to over-oxidation of aldehydes into their corresponding carboxylic acids,[9] we opted for the use of an alcohol dehydrogenase

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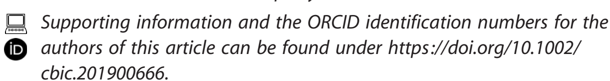

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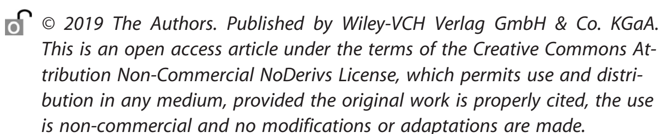

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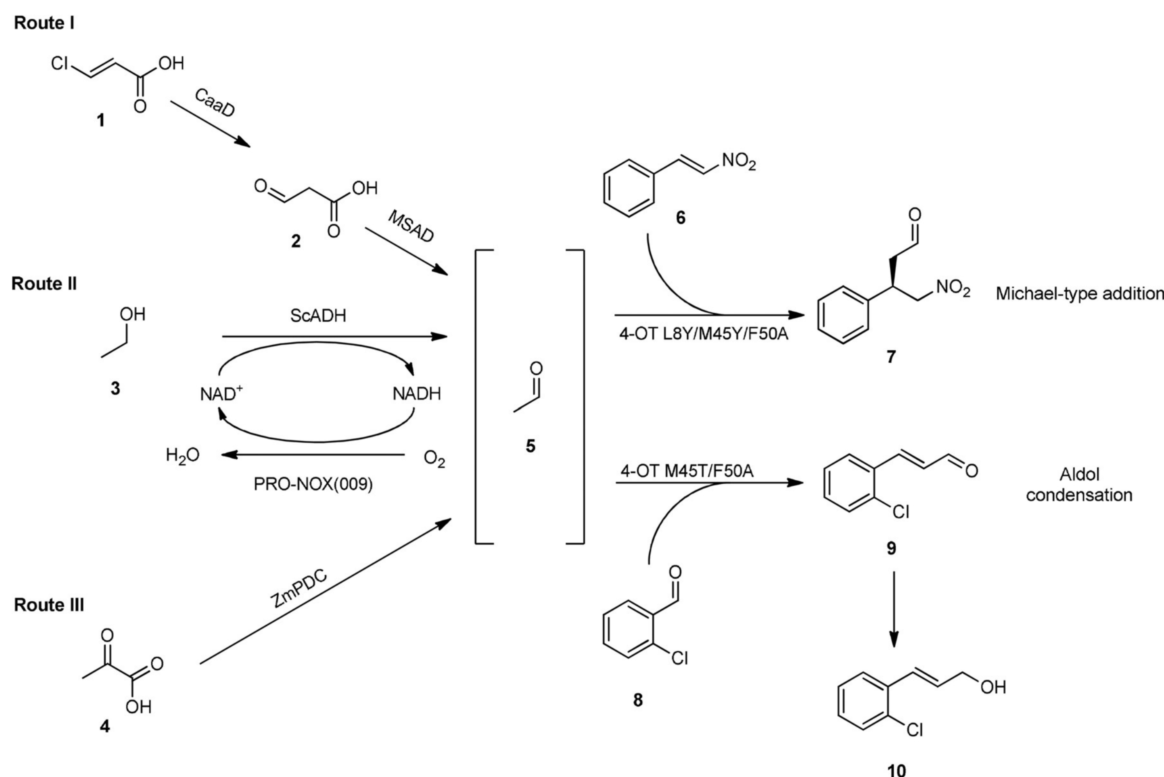

Scheme 1. Three envisioned enzymatic routes for in situ generation of acetaldehyde (**5**). The in situ synthesized **5** is used as substrate in a 4-OT L8Y/M45Y/F50A catalyzed Michael-type addition and a 4-OT M45T/F50A catalyzed aldol condensation reaction in one pot. Abbreviations: CaaD: chloroacrylic acid dehalogenase, MSAD: malonate semialdehyde decarboxylase, *Sc*ADH: alcohol dehydrogenase, PRO-NOX(009): NADH oxidase, *Zm*PDC: pyruvate decarboxylase.

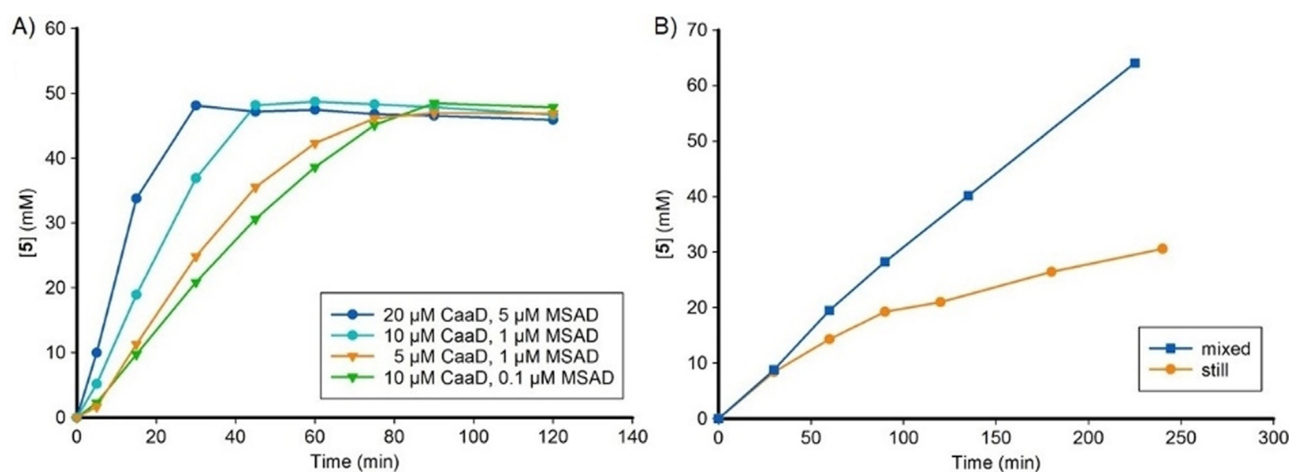

Figure 1. Progress curves of enzymatic synthesis of **5** using route I and route II. A) Formation of **5** via route I. Assay conditions: 50 mM **1**, 100 mM sodium phosphate (pH 7.3). B) Formation of **5** via route II. Assay conditions: 100 mM sodium phosphate pH 7.3, 2 mg mL^{-1} PRO-NOX(009), 10 U mL^{-1} ScADH, 10% *v*/*v* **3**, 2 mM NAD$^+$, 5 mL reaction volume, under oxygen atmosphere, performed in a 25 mL flask.

instead. The commercially available yeast alcohol dehydrogenase, (*Sc*ADH, *S. cerevisiae*) was selected as biocatalyst because of its high selectivity towards short-chain primary alcohols.[10,11] The commercially available NADH oxidase PRO-NOX(009) was selected to recycle NAD$^+$ and to overcome the unfavorable reaction equilibrium.

Concentrations of up to 10% *v*/*v* **3** were well tolerated by both enzymes, but higher concentrations rapidly inactivated at least one of the enzymes. Vigorous stirring of the reaction mixture under an oxygen atmosphere significantly improved the rate of **5** production compared to an unstirred reaction (Figure 1). Reaction conditions were optimized to reach a concentration of approximately 50 mM **5** after 150 minutes.

Lastly, we focused on the enzymatic decarboxylation of **4** to **5** (Scheme 1, route III). The pyruvate decarboxylase from *Z. mobilis* (*Zm*PDC) was cloned, expressed and purified to homogeneity. However, we were unable to find conditions that afforded concentrations of **5** above 30 mM, due to either inactivation

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or inhibition of the enzyme by **5**. This concentration was considered too low for effective catalysis with 4-OT and hence we abandoned this route.

Having established two effective enzymatic routes for the in situ synthesis of **5**, we next focused our attention on the combination of these routes with 4-OT in one-pot multistep cascade reactions. We initially focused on the 4-OT catalyzed Michael-type addition of **5** to **6** yielding **7**, an important precursor for the γ-aminobutyric acid analogue phenibut.[4,12] As a catalyst, the previously engineered 4-OT L8Y/M45Y/F50A variant was selected.[13] Combination of 4-OT L8Y/M45Y/F50A and **6** with in situ synthesized **5** (via both routes I and II) afforded *R*-**7** in high enantiopurity (*er* up to 98:2) and good to excellent isolated yields (up to 96% compared to **6**; Table 1).

Next, we focused on combining route I and II with the 4-OT catalyzed aldol condensation of **5** with **8**, forming **9**. The previously engineered 4-OT M45T/F50A was used as a catalyst for this reaction.[16] Combination of route I with **8** and 4-OT M45T/F50A afforded **9** in good isolated yield (56% compared to **8**; Figure 2 and Table 2). Interestingly, the combination of route II with **8** and 4-OT M45T/F50A afforded a mixture of **9** (26% isolated yield compared to **8**) and cinnamyl alcohol **10** (28% isolated yield compared to **8**; Figure 2 and Table 2). These results demonstrate some exciting opportunities available for the combination of natural metabolic routes with unnatural carboligation reactions in one pot.

Table 1. Stepwise enzymatic cascade synthesis of **7**.

Route I[a] — Route II[b] — step I — step II

	Reaction time [min] Step I[c]	Step II[d]	*er*[e]	Isolated yield [%][f]
Route I	150	75	98:2	62
Route II	90	85	98:2	96

[a] Dehalogenation of **1** into **2** catalyzed by CaaD, followed by decarboxylation of **2** into **5** catalyzed by MSAD, followed by the Michael-type addition of **5** to **6** yielding **7** catalyzed by 4-OT L8Y/M45Y/F50A. The reaction mixture consisted of 50 mM **1**, 4 mM **6**, 1 μM MSAD, 5 μM CaaD, 56 μM 4-OT L8Y/M45Y/F50A, 100 mM sodium phosphate pH 7.3, 10% *v/v* ethanol. [b] Oxidation of **3** into **5** catalyzed by *Sc*ADH, followed by the Michael-type addition of **5** to **6** yielding **7** catalyzed by 4-OT L8Y/M45Y/F50A. PRO-NOX(009) was used for co-factor recycling. The reaction mixture consisted of 10% *v/v* **3**, 4 mM **6**, 10 U mL^{-1} *Sc*ADH, 2 mg mL^{-1} PRO-NOX(009), 2 mM NAD$^+$, 56 μM 4-OT L8Y/M45Y/F50A, 100 mM sodium phosphate pH 7.3. [c] Monitored by HPLC. [d] Monitored by UV spectroscopy. [e] Determined by GC with chiral stationary phase or derivatized into a cyclic acetal and determined by HPLC with a chiral stationary phase. The absolute configuration was determined by comparison to literature.[14,15] [f] Isolated yield compared to **6**.

Discussion

4-OT is unique in that it can use **5** as substrate for C–C bond-forming Michael-type additions to nitroolefin acceptors and for aldol condensations with benzaldehydes.[3,4] Previously, we have demonstrated that 4-OT can be combined with other biocatalysts and chemocatalysts to convert **6** and **8** into GABA analogues using two complementary one-pot cascade reactions.[13,15] However, both routes rely on **5** as a substrate. The inherent high reactivity and toxicity of **5** makes it a less desirable substrate and special care has to be taken to prevent polymerization or oxidation. A solution to this problem is the enzymatic in situ generation of **5** using less toxic and reactive starting substrates in multistep cascade reactions. To this end, we have investigated three enzymatic routes for the in situ generation of **5**, using different starting materials.

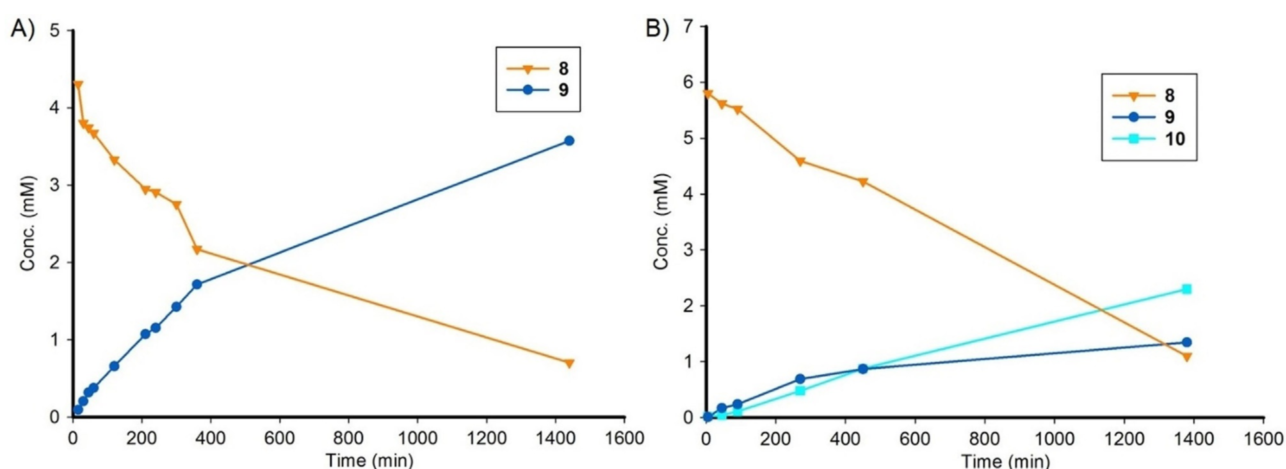

Figure 2. Progress curves of the enzymatic cascade synthesis of **9** and **10**. A) Progress curve of the 4-OT M45T/F50A catalyzed aldol condensation of **5** with **8** using in situ synthesized **5** via route I. Assay conditions: 50 mM **1**, 4 mM **8**, 1 μM MSAD, 10 μM CaaD, 75 μM 4-OT M45T/F50A, 150 mM sodium phosphate pH 7.3, 5% *v/v* ethanol, 17.8 mL reaction volume. B) Progress curve of the 4-OT M45T/F50A catalyzed aldol condensation of **5** with **8** using in situ synthesized **5** via route II. Assay conditions: 10% *v/v* **3**, 5.7 mM **8**, 2 mM NAD$^+$, 133 μM 4-OT M45T/F50A, 10 U mL^{-1} *Sc*ADH, 2 mg mL^{-1} PRO-NOX(009), 100 mM sodium phosphate pH 7.3, 30 mL reaction volume.

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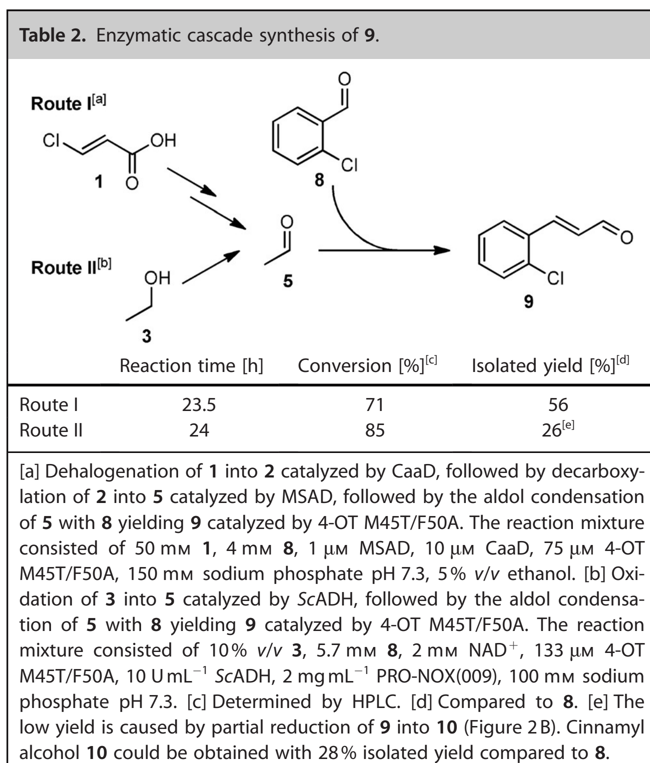
Table 2. Enzymatic cascade synthesis of **9**.



	Reaction time [h]	Conversion [%][c]	Isolated yield [%][d]
Route I	23.5	71	56
Route II	24	85	26[e]

[a] Dehalogenation of **1** into **2** catalyzed by CaaD, followed by decarboxylation of **2** into **5** catalyzed by MSAD, followed by the aldol condensation of **5** with **8** yielding **9** catalyzed by 4-OT M45T/F50A. The reaction mixture consisted of 50 mM **1**, 4 mM **8**, 1 μM MSAD, 10 μM CaaD, 75 μM 4-OT M45T/F50A, 150 mM sodium phosphate pH 7.3, 5% *v/v* ethanol. [b] Oxidation of **3** into **5** catalyzed by *Sc*ADH, followed by the aldol condensation of **5** with **8** yielding **9** catalyzed by 4-OT M45T/F50A. The reaction mixture consisted of 10% *v/v* **3**, 5.7 mM **8**, 2 mM NAD^+, 133 μM 4-OT M45T/F50A, 10 U mL^{-1} *Sc*ADH, 2 mg mL^{-1} PRO-NOX(009), 100 mM sodium phosphate pH 7.3. [c] Determined by HPLC. [d] Compared to **8**. [e] The low yield is caused by partial reduction of **9** into **10** (Figure 2B). Cinnamyl alcohol **10** could be obtained with 28% isolated yield compared to **8**.

The first route we investigated involves the conversion of soil pollutant **1** into **5**. Recycling of **1**, a degradation product from the xenobiotic soil fumigant *trans*-1,3-dichloropropene,[17] into GABA precursors provides an interesting example of the first principle of circular chemistry and as such might be desirable for future sustainable synthesis.[18] To this end, we selected the enzymes *trans*-3-chloroacrylic acid dehalogenase and malonate semialdehyde decarboxylase, which are part of the metabolic pathway for the degradation of *trans*-1,3-dichloropropene in *Pseudomonas cichorii* 170.[19,20] The second route comprises of the oxidation of **3** into **5**. As an inexpensive bio-renewable, **3** is an attractive precursor of **5** that can concurrently act as a co-solvent to solubilize **6** and **8**. To catalyze the oxidation of **3**, we selected yeast alcohol dehydrogenase, using an NADH oxidase for co-factor recycling and as a driving force to overcome the unfavorable reaction equilibrium between **3** and **5**. Thirdly, we investigated the readily available substrate **4** as precursor for **5**, using pyruvate decarboxylase from *Z. mobilis* as biocatalyst.

Gratifyingly, concentrations of 50 mM **5** or higher could be generated in situ using both route I and II. Route III only afforded lower concentrations of **5** and unfortunately, we were unable to find conditions to improve this. We speculate that *Zm*PDC was either inactivated or strongly inhibited by **5**,[21] and the effective use of route III for in situ generation of **5** awaits the discovery of 4-OT mutants with a lower K_M for **5**.

We demonstrated the modularity of our approach by showing that the in situ generated **5** can be used as a substrate for both 4-OT catalyzed aldol condensations and Michael-type additions. The Michael-type addition of **5** to **6** was catalyzed by the previously engineered 4-OT L8Y/M45Y/F50A and afforded *R*-**7** in good to excellent isolated yield (up to 96% compared to **6**) and with an excellent *er* value of 98:2. The aldol condensation of **5** with **8** was catalyzed by the previously engineered 4-OT M45T/F50A. Route I afforded **9** in 55% isolated yield, but route II afforded a mixture of **9** and **10**. The reduction of **9** to **10** is slowly catalyzed by *Sc*ADH and by an alcohol dehydrogenase present in the CFE of PRO-NOX(009).

Conclusion

In summary, we have investigated three enzymatic synthesis routes, using the readily accessible starting materials **1**, **3** or **4**, for in situ generation of **5** to be used as substrate by 4-OT in one-pot cascade syntheses. Route I and route II afforded **5** in effective concentrations for synthesis with 4-OT, but route III afforded **5** only in low concentrations. Route I and route II are fully compatible with 4-OT, which was demonstrated by the one-pot cascade synthesis of **7** and **9**. These modular enzymatic cascades provide a stepping stone towards the development of larger "artificial metabolisms" that could facilitate greener and more sustainable synthesis.[22]

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: acetaldehyde · biocatalysis · carboligation · cascade reactions

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