# Characteristics of an RNA Diels−Alderase Active Site

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**Abstract:** The active site components and substrate specificity of an RNA Diels−Alderase (DA22) were investigated. Diels−Alderase activity was found to be highly dependent on a unique 5-position pyridyl modified uridine. Even closely related pyridyl modifications failed to yield active catalysts. Substrate specificity of this Diels−Alderase was remarkable. Experiments with alternative diene and dienophile substrates showed the active site of the Diels−Alderase to be highly discriminating, even against molecules of similar reactivity and structure. Inhibition studies with a series of product analogues established that the RNA recognizes functional components in and around the reaction center of both the diene and the dienophile. Taken together, these results suggest that DA22 can fold into a structure that produces an intricate metal/ligand dependent active site capable of highly specific molecular recognition.

## Introduction

Among the most powerful of all synthetic organic transformations is the Diels−Alder [4 + 2] cycloaddition.[1] Despite the success of employing the Diels−Alder reaction to prepare biologically active carbocycles,[2] no naturally occurring Diels−Alderases have been isolated and fully characterized.[3] Recently, our group reported on the in vitro selection and characterization of the first RNA Diels−Alderase.[4] Unlike any of the known Diels−Alderase catalytic antibodies,[5] or any other known RNA catalyst,[6] the RNA Diels−Alderase showed a specific requirement for cupric ion and pyridyl substituents.[4] This Diels−Alderase represents a new mode of catalysis for this important class of cycloaddition reaction.[7] Understanding the specific requirements of the catalyst and substrates will reveal important new insights and could enhance our understanding of the mechanistic possiblities for this [4 + 2] cycloaddition.[8]

Considerable effort has been devoted to the discovery of new catalysts for Diels−Alder cycloadditions, with the primary objective being to enhance reactivity or control selectivity.[9] From a biological perspective, a key feature in any enzymatic process is substrate selectivity. For a Diels−Alderase, selectivity could be observed for either the diene or the dienophile and ideally both. Even with the immense amount of Diels−Alder catalyst research, on either macromolecules or small molecules, discrimination between dienophile substrates of similar reactivity has rarely been observed. Herein we describe the first examples of substrate selectivity by an RNA Diels−Alderase and the results from probing the active site through inhibition with a series of product analogues. In addition, the importance of the RNA modification[10] was investigated through either molecular replacement or rearrangement of the nitrogen contained in the pyridyl modified uridines.

## Results and Discussion

The discovery of RNA Diels−Alderases came from in vitro selection[4] on large libraries (ca. $10^{14}$ sequences) of 5-(4-methylpyridylcarboxamide)uridine[10c] modified RNA. Isolated sequence families were identified that catalyze the Diels−Alder cycloaddition shown in Scheme 1. One RNA from the largest family of Diels−Alderases, DA22, was characterized in detail

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**Scheme 1**

dsDNA Isolate 22

1) Transcription with ATP, CTP, GTP, U*TP
2) Ligate on diene-PEG-DNA 10-mer

U* = ; R = 3, 4, 5, 6, 7 (H)

R' = ribose-5'-triphosphate

and demonstrated a rate acceleration of 800-fold over the spontaneous reaction rate and an absolute dependence on the presence of cupric ion. The absolute dependence of catalytic activity on the presence of copper and the inability of isostructural metals to substitute for copper are highly suggestive of a Lewis acid role for the bound copper in DA22.[11] The DA22 sequence was also found to require the 5-(4-methylpyridylcarboxamide)uridine modification (**4** of Scheme 1) for catalytic activity.[4]

**Pyridine Replacement and Rearrangement.** To further define the structural requirements for catalytic activity and to establish the importance of the modified uridine, molecular replacement experiments were conducted. The DA22 sequence was transcribed[12] with each of the alternative UTP's shown in Scheme 1 (**3**, **5**–**7**) and the activity of each sequence evaluated (see the Experimental Section for details). The DA22 sequence transcribed with UTP **7** demonstrated no Diels–Alderase activity. This is not surprising given the drastic loss of functionality, both in hydrogen bonding and metal coordination, relative to the selected Diels–Alderase. However, transcription with the benzyl amide modified UTP **3** also gave RNA that was catalytically inactive. The functionality of **3** is identical to that of **4**, except the pyridyl heterocyclic N is replaced by the phenyl C–H. This result establishes an important role for the pyridine in catalytic activity. Further, the precise orientation of the pyridine nitrogen relative to the uridine ring was shown to be critical for activity, as shown by the inactivity of the sequences transcribed with either the 2- or the 3-methylpyridyl modified UTP's (**5** and **6**). When considered in the context of the copper-dependent activity, these results suggest that DA22 has a unique cupric ion binding site comprised of one or more pyridine ligands, which could serve as a Lewis acid to catalyze the Diels–Alder cycloaddition. It appears that this RNA Diels–Alderase can form a unique metal coordination geometry at the active site; however, these results do not reveal if specific substrate binding pockets are formed.

**Substrate Specificity.** To explore the Diels–Alderase 22 (DA22) active site, the relative catalytic activities for alternative substrates were determined as shown in Table 1 (**1**, **8**–**12**). Dienophile specificity was investigated using the acyclic diene **2**, while activity in the prescence of the alternative diene **12**

**Table 1**[a]

| Substrate | Structure | % Reacted after 3 min* |
|---|---|---|
| 1 | | 24.0 |
| 8 | | < 0.1‡ |
| 9 | | < 0.1‡ |
| 10 | | 0.96 |
| 11 | | 01.45 |
| 12 | N~PEG~DNA DA22 –3' | < 0.1‡ |

[a] Asterisk (*) indicates the values are background corrected and are relative to the total amount of active catalyst. Dagger (‡) indicates values below the assay detection limits.

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**Table 2**

| Compound | Product | $K_{iapp}$ μM |
|---|---|---|
| 13 | | 32.5 ± 2.6 |
| 14 | | 2300 ± 480 |
| 15 | | 1093 ± 150 |
| 16 | | 77.4 ± 5.3 |

was determined using maleimide **1**. The extent of reaction was determined after 3 min of incubation as described in the Experimental Section. It should be noted that the spontaneous cycloaddition rate for dienophiles **1**, **8**, **10**, and **11** with dienes **2** or **12** gave relative rates within a factor of 2 of each other. Therefore, the inherent reactivity of these diene and dienophile substrates cannot be a determining factor in the substrate specificity of DA22.[13] If DA22 was simply acting as a general Lewis acid, it might be expected that dieneophile **1** would react with the cyclic diene **12** at a rate approaching that of the Diels–Alderase substrates **1** and **2**. Similarly, fumarate dienophile **8** reacting with diene **2** would also be expected to benefit from general Lewis acid catalysis. Remarkably, DA22 showed surprising selectivity, giving no rate enhancement over background for either **1** reacting with **12** or **8** reacting with **2**. Even more stunning, DA22 displayed a distinct selectivity against the very similar dienophiles **10** and **11** reacting with **2**. Both **10** and **11** are maleimide dienophiles and only begin to differ at the second carbon center distal to the maleimide nitrogen. The observed selectivity for DA22 was 100:6:4 for **1**:**10**:**11** reacting with **2**. Not only are these alternative substrates identical to **1** electronically, but the functionalities attached to the maleimides are similar and, for **11,** less sterically demanding. Clearly, the active site of this catalytic RNA has an inherent ability to discriminate between very similar substrates and the Diels–Alderase activity observed can be ascribed to much more than general Lewis acid catalysis.

**Product Inhibition.** To further survey the molecular recognition capabilities of the DA22 active site and access the contribution of individual functional components within the diene and dienophile, a product inhibition study was undertaken.[14] The apparent inhibition constant ($K_{iapp}$) was determined for the series of cycloaddition products shown in Table 2. Diels–Alderase 22 inhibition by **13** was previously reported,[4] and since it contains all of the functionality present in the diene and dienophile, its $K_{iapp}$ value served as a benchmark by which to measure the other inhibitors in the series. The minimal product, **14**, which only presented the core functionality of the diene and dienophile, displayed the weakest inhibition ($K_{iapp}$ = 2.3 mM). As functional groups from both the diene and the dienophile were restored, inhibition of DA22 catalysis increased, as demonstrated by the $K_{iapp}$ values for compounds **15** and **16** (Table 2). This trend clearly demonstrates that the RNA recognizes functional group components of both the diene and the dienophile. Furthermore, there is only a 2-fold difference between the inhibition constants measured for **13** and **16**, suggesting that the functionality remote to the cyclohexane, including the biotin substituent, does not contribute significantly to the dienophile substrate binding. The product inhibition data, in combination with the substrate specificity observed for DA22, demonstrate that the active site recognizes substrate structure surrounding the [4 + 2] reaction center, as well as attached functionality.

(13) Only dieneophile **9** gave a significantly slower rate for the spontaneous Diels–Alder reaction with **2**.

(14) Because of the product-like transition state for Diels–Alder cycloaddition, product analogues can serve as useful inhibitors.

## Conclusions

It is now known that RNA Diels–Alderase 22 requires specific pyridyl-uridine modifications to the extent that even rearrangement of the pyridine nitrogen results in complete loss of catalytic activity. These modifications are proposed to provide unique cupric ion coordination environments. DA22 is an intricate macromolecular catalyst that depends on the precise presentation of its own functional groups for Diels–Alderase activity. Furthermore, the active site created by this modified RNA catalyst dictates precise interactions with both substrates,[15] ultimately leading to high substrate selectivity.

This is the first example of RNA exhibiting substrate selectivity in a bimolecular transformation of small molecule substrates. Taken together, these results indicate that DA22 can fold in such a way as to create a sensitive and discriminating active site analogous to protein enzymes. In fact, the substrate molecular recognition of this RNA catalyst rivals its protein counterparts.[5] These results are surprising when considering the structural and electronic differences in proteins versus nucleic acids. Within DA22 and other isolated Diels–Alderase structures lies a wealth of information, which will improve our limited understanding of oligonucleotide catalysts. Moreover, structural studies on these new catalysts may be informative with regard to the mechanism of Lewis acid catalysis of this important cycloaddition reaction. Research is currently underway to reveal the genetic, structural, and functional relationships that exist within families of Diels–Alder catalysts.

## Experimental Section

**Molecular Replacement/Rearrangment Experiments.** 5-Position modified uridine nucleosides were synthesized using a previously published procedure.[16] Modified nucleotide triphosphates **3**–**6** were prepared according to the protocol of Whitesides.[17] Analytical data for 5′-monophosphate uridines, precursors to the 5′-triphosphates, **3**–**6** are shown below. Detailed experimental procedures for the synthesis of these compounds will be reported elsewhere. RNA Diels–Alder reaction incubation were conducted as described previously at 500 μM **1**.[4] All modified nucleosides were characterized spectroscopically as the nucleotide monophosphates.

**3:** $^{1}$H NMR ($D_2O$) δ 4.12 (m, 2 H), 4.30 (m, 2 H), 4.39 (t, $J$ = 4.7 Hz, 1 H), 4.53 (s, 2 H), 5.93 (d, $J$ = 5.0 Hz, 1 H), 7.30 (m, 5 H), 8.55 (s, 1 H); $^{13}$C NMR ($D_2O$:MeOD = 4:1) δ 44.0, 65.4, 71.0, 75.3, 85.0,

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90.7, 106.8, 128.2, 128.5, 129.8, 147.4, 151.5, 164.5, 164.6; HRMS (FAB+) *m/z* 719.1488 (M (triphosphate) + $Et_3NH^+$, calcd = 719.1496 for $C_{23}H_{38}N_4O_{16}P_3$).

**4:** $^1H$ NMR ($D_2O$) $\delta$ 4.0 (m, 2 H), 4.23 (m, 1 H), 4.28 (t, $J$ = 4.5 Hz, 1 H), 4.43 (t, $J$ = 5.2 Hz, 1 H), 4.60 (s, 2 H), 5.93 (d, $J$ = 5.1 Hz, 1 H), 7.34 (d, $J$ = 4.8 Hz, 2 H), 8.42 (d, $J$ = 4.8 Hz, 2 H), 8.55 (s, 1 H); $^{13}C$ NMR ($D_2O$:MeOD = 100:1) $\delta$ 43.2, 64.9, 71.1, 74.9, 85.1, 90.7, 106.9, 123.4, 147.7, 149.4, 150.2, 152.1, 165.0, 165.5; $^{31}P$ NMR (MeOD) $\delta$ 8.2; HRMS (FAB+) *m/z* 459.0909 (M + $H^+$, calcd = 459.0917 for $C_{16}H_{20}N_4O_{10}P$).

**5:** $^1H$ NMR ($D_2O$) $\delta$ 4.0 (m, 2 H), 4.25 (m, 2 H), 4.39 (t, $J$ = 4.8 Hz, 1 H), 4.52 (s, 2 H), 5.89 (d, $J$ = 4.9 Hz, 1 H), 7.38 (dd, $J$ = 7.7, 5.1 Hz, 1 H), 7.75 (d, $J$ = 7.9 Hz, 1 H), 8.36 (d, $J$ = 4.7 Hz, 1 H), 8.42 (s, 1 H), 8.50 (s, 1 H); $^{13}C$ NMR ($D_2O$:MeOD = 100:1) $\delta$ 43.3, 65.0, 71.1, 74.9, 85.1, 90.8, 106.9, 125.4, 135.5, 137.7, 147.6, 148.4, 151.9, 165.0; $^{31}P$ NMR (MeOD) $\delta$ 2.2; HRMS (FAB+) *m/z* 459.0913 (M + $H^+$, calcd = 459.0917 for $C_{16}H_{20}N_4O_{10}P$).

**6:** $^1H$ NMR ($D_2O$) $\delta$ 3.98 (m, 2 H), 4.23 (m, 2 H), 4.41 (t, $J$ = 5.1 Hz, 1 H), 4.62 (s, 2 H), 5.91 (d, $J$ = 5.1 Hz, 1 H), 7.31 (dd, $J$ = 7.4, 5.6 Hz, 1 H), 7.80 (td, $J$ = 7.8, 1.7 Hz, 1 H), 8.42 (d, $J$ = 4.7 Hz, 2 H), 8.51 (s, 1 H); $^{13}C$ NMR ($D_2O$:MeOD = 100:1) $\delta$ 43.5, 65.7, 72.0, 76.0, 85.6, 91.4, 106.8, 123.1, 124.0, 139.0, 147.6, 150.0, 152.5, 159.0, 165.0,165.9; $^{31}P$ NMR (MeOD) $\delta$ 3.7; HRMS (FAB+) *m/z* 459.0909 (M + $H^+$, calcd = 459.0917 for $C_{16}H_{20}N_4O_{10}P$).

**Alternative Substrate Experiments.** Biotin Maleimides **1** and **10** were purchased from Pierce while compound **11** was purchased from Sigma. All were used without further purification. Diene **12** was prepared and characterized as previously described.[18] Fumarate **8** and methacrylamide **9** were synthesized as described below. Incubations were conducted as described previously[4] at 500 $\mu$M dienophile, 500 nM RNA, and 25 °C.

**8.** Maleic anhydride (10.2 mmol) and 2-methoxyethanol (6.3 mmol) were combined and heated to 100 °C for 18 h. The reaction mixture was then added to 100 mL of hexanes and agitated, and the hexane layer was decanted. The remaining oily residue was washed once more with hexanes (100 mL) and then refluxed for 2 h with thionyl chloride (12.7 mmol). The excess thionyl chloride was then removed under reduced pressure, and the desired acid chloride was added dropwise over 30 min to a solution of *N*-hydroxysuccinimde (12.7 mmol) and triethylamine (12.7 mmol) in 30 mL of anhydrous THF at 0 °C. The reaction mixture was allowed to warm to ambient temperature for 2 h followed by removal of the THF by rotary evaporation and addition of diethyl ether (100 mL). The oraganic mixture was washed with brine (3 × 75 mL) and dried over $MgSO_4$. The product was purified by flash silica gel chromatography to give a 65% yield of the NHS fumarate ester. The fumarate ester (1.05 mmol), *N*-(2-aminoethyl)biotinamide (1.2 mmol, Molecular Probes), and triethylamine (1.2 mmol) were combined in 5 mL of ethanol at ambient temperature. The mixture was stirred for 18 h followed by removal of the solvent under reduced pressure. The remaining white residue was triturated with ethyl acetate (10 × 5 mL) to give a 67% yied of the desired biotinylate fumaramide **8:** $^1H$ NMR (MeOD) $\delta$ 1.41–1.60 (m, 6 H), 2.19 (t, $J$ = 7.5 Hz, 2 H), 2.69 (d, $J$ = 12.9 Hz, 1 H), 2.91 (dd, $J$ = 12.9, 5.0 Hz, 1 H), 3.37 (m, 7 H), 3.63 (t, $J$ = 4.7 Hz, 2 H), 4.31 (m, 3 H), 4.46 (m, 1 H), 6.71 (d, $J$ = 15.5 Hz, 1 H), 7.00 (d, $J$ = 15.5 Hz, 1 H); $^{13}C$ NMR (MeOD) $\delta$ 26.8, 29.5, 29.8, 36.8, 39.8, 40.4, 41.1, 57.0, 59.1, 61.7, 63.4, 65.2, 71.4, 130.6, 138.0, 166.2, 166.4, 166.8, 176.5; HRMS (FAB+) *m/z* 443.1968 (M + $H^+$, calcd = 443.1964 for $C_{19}H_{32}N_4O_6S$).

**9.** To a solution of *N*-hydroxysuccinimide (5.2 mmol) and triethylamine (5.2 mmol) in 13 mL of anyhydrous THF at 0 °C was added dropwise over 30 min methacrylyl chloride (5.2) in 13 mL of THF. The reaction was allowed to warm to room temperature over 2 h followed by removal of the THF under reduced presuure. The remaining residue was dissolved in diethyl ether (100 mL), washed with brine (3 × 75 mL), and dried over $MgSO_4$. The resulting NHS ester was purified by flash silica gel chromatography (62%), and 1.4 mmol was dissolved in 28 mL of anhydrous THF, cooled to 0 °C, and treated with ethylenediamine (20 mmol). The reaction mixture was allowed to warm to ambient temperature overnight. A white precipitate was removed and the filtrate concentrated by rotary evaporation. Continued evaporation under high vacuum gave the desired monoamide/monamine in 90% yield from the NHS ester. The methacroylyl amine (161 mg, 1.26 mmol) was then combined with NHS–biotin (357 mg, 1.05 mmol) in 5 mL of ethanol. The heterogeneous mixture was stirred at ambient temperature for 18 h followed by removal of the ethanol under reduced pressure. The remaining white residue was triturated with ethyl acetate (5 × 5 mL) to give compound **9** as a white solid in 42% yield from the biotin–NHS ester: $^1H$ NMR (DMSO-$d_6$) $\delta$ 1.30–1.60 (m, 6 H), 1.84 (s, 3 H), 2.05 (t, $J$ = 7.4 Hz, 2 H), 2.56 (d, $J$ = 12.4 Hz, 1 H), 2.81 (dd, $J$ = 12.4, 5.07 Hz, 1 H), 3.13 (m, 5 H), 4.13 (m, 1 H), 4.29 (m, 1 H), 5.32 (s, 1 H), 5.65 (s, 1 H), 6.37 (s, 1 H), 6.44 (s, 1 H), 7.88 (s, 1 H), 7.93 (s, 1 H); $^{13}C$ NMR (DMSO-$d_6$) $\delta$ 18.6, 25.3, 28.1, 28.2, 35.2, 38.0, 38.1, 45.7, 55.4, 59.1, 60.9, 119.2, 139.8, 162.7, 167.5, 172.4; HRMS (FAB+) *m/z* 355.1810 (M + $H^+$, calcd = 355.1804 for $C_{16}H_{28}N_4O_3S$).

**Diels–Alderase Inhibition by Product Analogues.** The Diels–Alder products were synthesized from the appropriate diene and maleimide as described in the following general procedure. The diene (4.62 mmol) and maleimide (4.62 mmol) were dissolved in 10 mL of benzene and heated to 45 °C for 2–7 days. The reaction mixture was then evaporated to dryness and the cycloaddition product purified by flash silica gel chromatography using hexane/ethyl acetate at the eluent. Products were analyzed by $^1H$ NMR, $^{13}C$ NMR, and MS.

**14:** $^1H$ NMR ($CDCl_3$) $\delta$ 1.07 (t, $J$ = 7.2 Hz, 3 H), 1.48 (d, $J$ = 7.2 Hz, 3 H), 2.40 (m, 1 H), 2.56 (m, 1 H), 3.03 (dd, $J$ = 8.8, 6.5 Hz, 1 H), 3.32 (m, 2 H), 3.45 (q, $J$ = 7.2 Hz, 2 H), 3.95 (m, 2 H), 5.67 (dt, $J$ = 9.1, 3.1 Hz, 1 H), 5.73 (dt, $J$ = 9.1, 2.8 Hz, 1H); $^{13}C$ NMR ($CDCl_3$) 13.5, 17.3, 32.2, 34.5, 40.0, 43.9, 46.4, 63.3, 131.1, 135.9, 180.4, 180.5; HRMS (FAB+) *m/z* 224.1284 (M + $H^+$, calcd = 224.1286 for $C_{12}H_{18}NO_3$).

**15:** $^1H$ NMR ($CDCl_3$) $\delta$ 0.90 (m, 2 H), 1.15 (m, 3 H), 1.49 (d, $J$ = 7.2 Hz, 3 H), 1.50–1.78 (m, 7 H), 2.45 (m, 1 H), 2.60 (m, 1 H), 3.05 (dd, $J$ = 8.5, 6.5 Hz, 1 H), 3.30 (d, $J$ = 6.9 Hz, 2 H), 3.34 (dd, $J$ = 8.5, 7.2 Hz, 1 H), 3.90 (dd, $J$ = 12.0, 9.4 Hz, 1 H), 4.02 (dd, $J$ = 12.0, 5.7 Hz, 1 H), 5.70 (dt, $J$ = 9.1, 3.1 Hz, 1 H), 5.76 (dt, $J$ = 9.1, 3.1 Hz, 1 H); $^{13}C$ NMR ($CDCl_3$) $\delta$ 16.8, 25.5, 26.1, 30.6, 30.6, 31.2, 36.1, 38.1,42.7, 44.7, 62.9, 128.4, 135.3, 177.7, 179.3; HRMS (FAB +) *m/z* 292.1920 (M + $H^+$, calcd = 292.1912 for $C_{17}H_{26}NO_3$).

**16:** $^1H$ NMR ($CDCl_3$) $\delta$ 0.88 (m, 2 H), 1.13 (m, 3 H), 1.44 (d, $J$ = 7.4 Hz, 3 H), 1.48–1.73 (m, 7 H), 2.43 (m, 1 H), 2.60 (m, 1 H), 3.01 (dd, $J$ = 8.5, 7.1 Hz, 1 H), 3.20 (dd, $J$ = 8.7, 6.0 Hz, 1 H), 3.27 (d, $J$ = 6.9 Hz, 2 H), 3.40 (broad q, $J$ = 5.2 Hz, 2 H), 3.57 (t, $J$ = 5.0 Hz, 4 H), 3.73 (t, $J$ = 4.9 Hz, 2 H), 4.51 (dd, $J$ = 11.0, 8.1 Hz, 1 H), 4.63 (dd, $J$ = 11.0, 7.4 Hz, 1 H), 5.25 (broad t, 1 H), 5.74 (m, 2 H); $^{13}C$ NMR ($CDCl_3$) $\delta$ 16.6, 25.5, 26.2, 30.6, 31.3, 36.1, 36.2, 40.8, 42.2, 44.5, 44.6, 61.7, 64.9, 70.0, 72.2, 129.2, 135.2, 156.5, 177.4, 177.5; HRMS (FAB +) *m/z* 423.2505 (M + $H^+$, calcd = 423.2495 for $C_{22}H_{35}N_2O_6$).

**Product Inhibition.** Apparent $K_i$ values for the cycloaddition products **13**–**16** were determined at 500 $\mu$M **1** by fitting the observed first-order rate constants to the following equation for inhibition: $k_{obs} = (k_{obs0}/2)(\alpha E - I - K_i + ((K_i + \alpha E - I)^2 + 4K_i)^{1/2})$, where $k_{obs}$ is the measured rate constant in the presence of inhibitor, $k_{obs0}$ is the observed rate constant in the absence of inhibitor, $\alpha E$ represents the fractional ($\alpha$) concentration of functional active sites ($E$), $I$ is the concentration of inhibitor, and $K_i$ is the apparent inhibition constant.[19]

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