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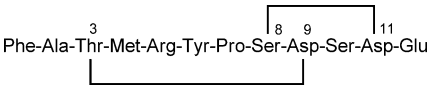
# Role of the Backbone Conformation at Position 7 in the Structure and Activity of Marinostatin, an Ester-Linked Serine Protease Inhibitor

Misako Taichi,[a] Toshimasa Yamazaki,[c] and Yuji Nishiuchi*[a, b]

Marinostatin (MST, Scheme 1), isolated from the marine organism *Pseudoaltermonus sagamiensis*, is a serine protease inhibitor that consists of 12 amino acids with two internal ester linkages formed between the β-hydroxyl and β-carboxyl groups, Thr3–Asp9 and Ser8–Asp11.[1–3] MST strongly inhibits subtilisin




**Scheme 1.** Primary structure of MST.

($K_i = 1.5$ nM), chymotrypsin, and elastase at an enzyme/inhibitor ratio of 1:1, but is not active against trypsin.[2] Its strong inhibitory potential is attributed to the internal hydrogen bond linking the backbone NH proton of Arg5 to the carbonyl oxygen atom of the Thr3–Asp9 ester linkage to protect the scissile Met4–Arg5 peptide bond.[3] Most serine protease inhibitors are composed of 14 to 190 amino acids, and bind to their cognate enzymes in a substrate-like manner. They have an exposed binding loop that possesses a characteristic canonical conformation stabilized mostly by crosslinked disulfide bridges, although global three-dimensional structures are not common.[4,5] MST is the smallest serine protease inhibitor from a natural source, and the structure of its reactive site, which is prescribed by the ester linkages, is considered to adopt the same canonical conformation as those of the typical serine proteases.

In a previous study,[6] we synthesized MST by regioselective esterification, employing two sets of orthogonally removable side-chain-protecting groups for Asp and Thr/Ser. The solution structure of MST revealed that the Ramachandran angles of the reactive site, MST(1–6), coincide with those of the binding loop of the turkey ovomucoid third domain (OMTKY3), a representative serine protease inhibitor.[7] In addition, a SAR study of MST indicated that the Thr3–Asp9 ester linkage, the conformation of *cis*-Pro7, and the N-terminal Phe1–Ala2 residues are essential for inhibitory activity. Of particular importance is that the *cis* conformation at Pro7 contributes to a rigid turn structure in Tyr6–Pro7 that promotes the internal hydrogen bond between Arg5 and the Thr3–Asp9 ester linkage, which not only protects the scissile bond but also maintains structural rigidity. Thus, it was expected that replacing the *cis* amide bond with a *trans* one would result in a loss of the potency. However, [Ala7]-MST was found to retain significant inhibitory activity, albeit two orders of magnitude less potent than the original level, even though it had the *trans* conformation at Ala7 in the enzyme-free solution structure. This conflicted with the strict substrate/inhibitor recognition of serine proteases, and so implied that the *trans* conformation at Ala7 might isomerize to the *cis* one that is responsible for expressing, even if only to a small extent, the canonical structure bound to proteases. To exclude the possibility of isomerization at Ala7, we designed the MST olefin analogue **1**, in which an *E* olefin is substituted for the amide of Tyr6–Ala7 so as to keep the *trans* backbone structure. In addition, *Z*-olefin analogue **2** was synthesized to confirm regeneration of the original inhibitory activity when the MST molecule adopts the *cis* conformation at position 7. The olefin frameworks of **1** and **2** were substituted for MST(6–7) as olefin dipeptide isosteres TyrΨ[(*E*)-CH=CH]Gly (**3**) and TyrΨ[(*Z*)-CH=CH]Gly (**4**), which were prepared by employing a modified Julia–Kocieński olefination[8] and a Wittig olefination, respectively. The synthesis of **3** commenced with the Mitsunobu reaction of the mono-TBS-protected (TBS = *tert*-butyldimethylsilyl) alcohol **5** and the thiol **6** followed by molybdenum-mediated oxidation[9] of the resulting sulfide to give sulfone **7** (Scheme 2A). The modified Julia-Kocieński olefination of Boc-Tyr(Dcb)-H (**8**) and sulfone **7** proceeded smoothly to afford exclusively the desired *E* olefin unit **9** when KHMDS/DMF was used as solvent. The Fmoc derivative **3** was prepared by removal of the TBS group from **9** followed by a two-step oxidation and conversion of the $N^{\alpha}$ protecting group. To construct the *Z* olefin framework of **4**, a Wittig reaction was performed on the phosphonium salt **10** and the tyrosine aldehyde **8** (Scheme 2B). The *Z* olefin unit **11** was obtained with a high selectivity ($E/Z = 3:97$) in 69% yield by using NaHMDS in THF. However, oxidation of alcohol **12**, which is formed by removal of the TBS group, led to a mixture of by-products, probably due to interaction between the NH proton of the Tyr isostere and the resulting aldehyde, which are spatially localized in the *Z* configuration. To avoid this side reaction, another Boc group had to be introduced into the secondary amine of the Tyr isostere in order to perform oxidation of the alcohol **13** to the carboxylic acid **14** (83% yield for two steps), which was converted to the Fmoc derivative **4**. The olefin dipeptide isosteres **3** and **4** were clearly separated and no contamination by the other isomer in either product was observed by RP-HPLC; this

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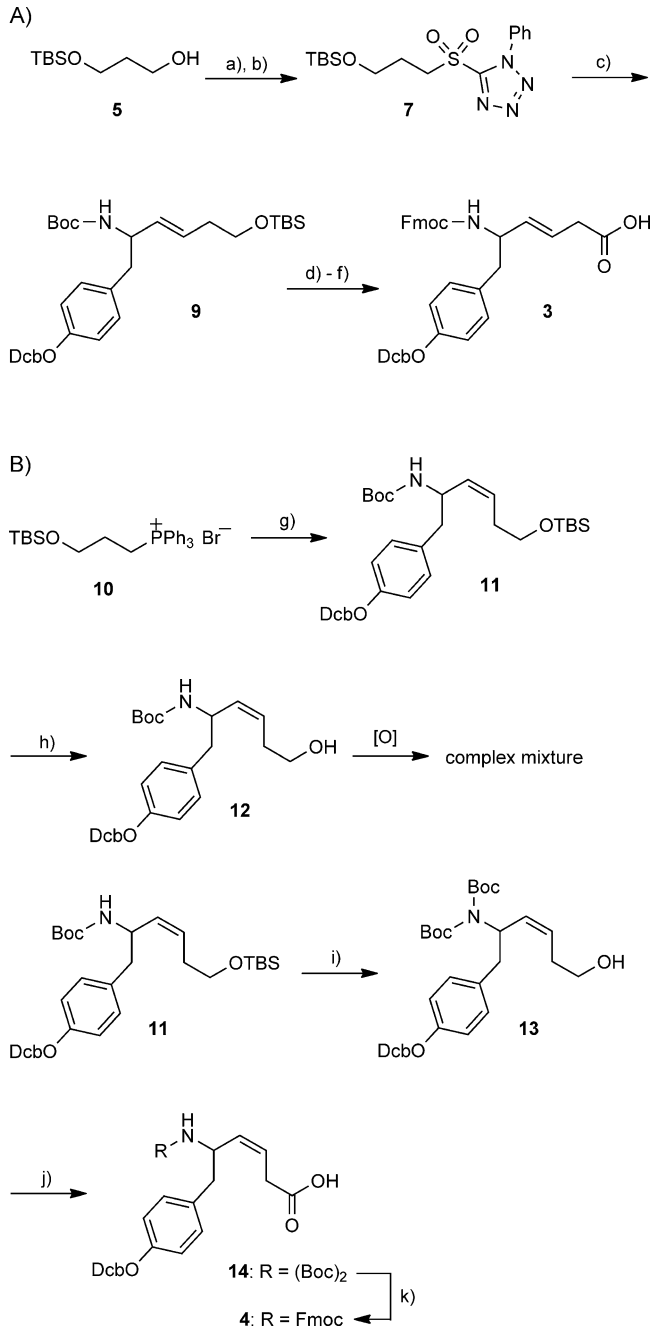
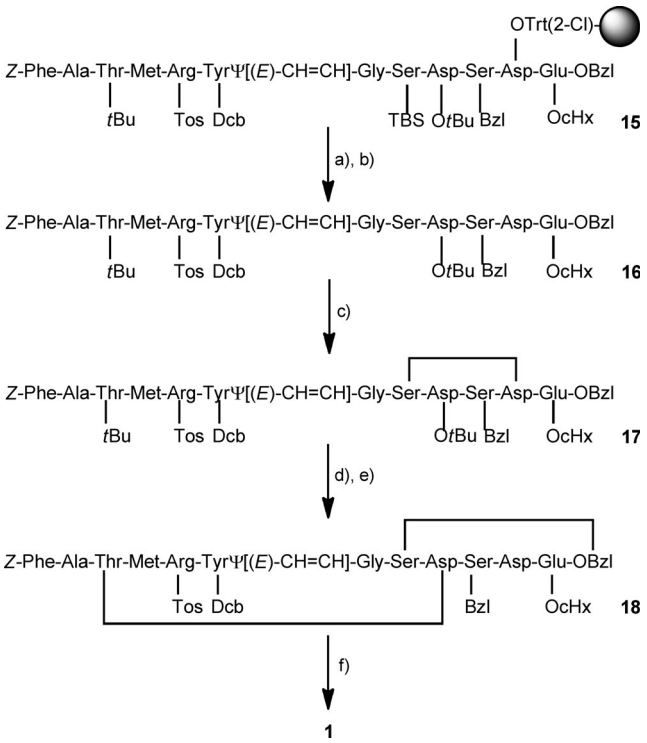



**Scheme 2.** Synthesis of A) *E* olefin dipeptide isostere **3** and B) *Z* olefin dipeptide isostere **4**. a) $PPh_3$, DIAD, 1-phenyl-1*H*-tetrazol-5-thiol (**6**), THF, 0 °C, 93%; b) $H_2O_2$ aq., $(NH_4)_6Mo_7O_{14}{\cdot}4H_2O$, EtOH, 0 °C, 10 h, 72%; c) Boc-Tyr(Dcb)-H (**8**), KHMDS, DMF, −78 to 0 °C, 2 h, 40%; d) TBAF, THF, RT, 50 min, 83%; e) i: DMP, $NaHCO_3$, $CH_2Cl_2$, 0 °C to RT, 35 min; ii: $NaClO_2$, $NaH_2PO_4$, DMSO/$H_2O$ (4:1, *v/v*), 0 °C to RT, 40 min, 69% for two steps; f) i: TFA, 0 °C to RT, 40 min; ii: Fmoc-OSu, DIEA, DMF/$H_2O$ (4:1, *v/v*), 0 °C to RT, 2 h, 89% for two steps; g) Boc-Tyr(Dcb)-H (**8**), NaHMDS, THF, −78 °C to 0 °C, 2.5 h, 69%; h) TBAF, THF, RT, 50 min, quant; i) i: $Boc_2O$, LiHMDS, THF, 0 °C, 40 min; ii: TBAF, THF, RT, 96% for two steps; j) i: DMP, $NaHCO_3$, $CH_2Cl_2$, 0 °C to RT, 1.5 h; ii: $NaClO_2$, $NaH_2PO_4$, DMSO/$H_2O$ (4:1, *v/v*), 0 °C to RT, 35 min, 83% for two steps; k) i: TFA, 0 °C to RT, 40 min; ii: Fmoc-OSu, DIEA, DMF/$H_2O$ (4:1, *v/v*), 0 °C to RT, 1.5 h. $Boc_2O$ = di-*tert*-butyl dicarbonate, Dcb = 2,6-dichlorobenzyl, DIAD = diisopropyl azadicarboxylate, DIEA = *N*,*N*-diisopropylethylamide, DMP = Dess–Martin periodinate, Fmoc-OSu = *N*-(9-fluorenylmethoxycarbonyloxy)succinimide, KHMDS = potassium bis(trimethylsilyl)amide, LiHMDS = lithium bis(trimethylsilyl)amide, NaHMDS = sodium bis(trimethylsilyl)amide, TBAF = tetrabutylammonium fluoride, TFA = trifluoroacetic acid.

indicates that both products possessed a high degree of isomeric purity. The starting linear protected peptide (**15**) for the *E* olefin analogue was assembled by solid-phase peptide synthesis with Fmoc chemistry onto the C-terminal dipeptide, which was attached to a Trt(2-Cl) resin through the β-carboxyl group of Asp11 as reported previously (Scheme 3).[6] Peptide




**Scheme 3.** Synthesis of *E* olefin analogue **1**. a) HFIP/$CHCl_3$ (4:1, *v/v*), RT, 1 h, 79%; b) TASF, DMF, RT, 1 h, 95%; c) MNBA, DMAP, TEA, $CH_2Cl_2$/DMF (5:1, *v/v*), RT, 2.5 h, 89%; d) TFA, 0 °C to RT, 1 h, 96%; e) MNBA, DMAP, TEA, $CH_2Cl_2$/DMF (5:1, *v/v*), RT, 3 h, 88%; f) HF/*p*-cresol (4:1, *v/v*), −5 to −2 °C, 1 h, 18%. cHx = cyclohexyl, HFIP = 1,1,1,3,3,3-hexafluoropropan-2-ol, MNBA = 2-methyl-6-nitrobenzoic anhydride, TEA = triethylamine, TASF = tris(dimethylamino)sulfonium difluorotrimethylsilicate.

chain elongation was carried out by using 1 min preactivation of coupling with Fmoc derivative/2-(6-chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU)/6-chloro-1-hydroxy-1*H*-benzotriazole (6-Cl-HOBt)/DIEA (4:4:4:8 equiv)—except for coupling of the *E* olefin dipeptide isostere **3**, which was incorporated by the *N*,*N*′-diisopropyl carbodiimide (DIC)/HOBt method because reaction mediated by HCTU/6-Cl-HOBt or PyBOP was accompanied by isomerization from the β,γ-unsaturated structure to the α,β-unsaturated one. After cleavage of the Trt(2-Cl) resin and the TBS group on Asp11 and Ser8, respectively, the resultant peptide, **16**, was subjected to intramolecular esterification by using Shiina reagent, 2-methyl-6-nitrobenzoic anhydride (MNBA),[10] to preferentially produce the cyclic monomer **17** in a 89% yield, with a small amount of by-products such as aspartimide (Asi) peptide formed at Asp11, and cyclic/linear dimers. The second intramolecular esterification with Thr3–Asp9 was performed by employing the same procedure with Ser8–Asp11. The diester

peptide was treated with HF in the presence of *p*-cresol at −5 to −2 °C for 1 h to remove all the protecting groups with no loss of the ester linkages, and the product **1** was obtained after purification by RP-HPLC. The *Z* olefin analogue **2** was synthesized by employing the same procedure as for **1**. Upon cyclization of the olefin analogues **1** and **2**, no significant differences were observed between the *E* and *Z* olefin dipeptide isosteres in terms of the efficiency of yielding individual ester linkages.

The solution structures of **1** and **2** as determined by NMR are shown in Figure 1. As we had expected, the $\phi$ and $\psi$ backbone dihedral angles of P2–P2′ in **2** were comparable to those in MST, whereas those in **1** were like those in [Ala7]-MST (Table 1). Comparison of the solution structure of **2** with the crystal structure of OMTKY3 bound to subtilisin Carlsberg (CARL; PDB ID: 1YU6)[11] revealed that the structures of the P4–P2′ regions of these two inhibitors superimposed well, with a root-mean-square deviation (RMSD) of 0.70 Å for the main-chain N, Cα and C atoms (Figure 2A). Furthermore, the side chain of Tyr6 in **2** adopted the same conformation as the corresponding Tyr20 in OMTKY3, which forms a hydrophobic stacking interaction with Phe189 in CARL. These structural similarities demonstrate that even in the free form, the *Z* olefin analogue **2** has the P4–P2′ structure preferentially organized to bind to CARL. In contrast, the P4–P2′ region of the *E* olefin analogue **1** has a significantly different structure, particularly in the Arg5(P1′)–Tyr6(P2′) region that assumes a tight βI-turn-like structure and might disrupt favorable interactions between the inhibitor and enzyme (Figure 2B). These observations clearly indicated that the *cis* backbone conformation at position 7 could play a critical role in prescribing the canonical structure for the reactive site of MST(P2–P2′). Thus, the *E* olefin analogue **1** completely lost inhibitory activity against subtilisin, whereas the *Z* olefin analogue **2** retained its potency to a substantial level (Table 2). It should be noted that subtilisin cannot interact with a MST analogue that possesses a *trans* conformation at position 7. These results demonstrated that isomerization at Ala7 of [Ala7]-MST can lead to binding to proteases in a canonical manner. The structure of the larger ring in MST, that is, MST(3–9), which is governed by the ester linkage with Thr3–Asp9 and *cis*-Pro7, is considered to be strengthened by the smaller ring MST(8–11). When the smaller ring of MST opened, isomerization took place at Pro7 to decrease the amount of the *cis* isomer to 85%; this lowered the potency ($K_i$ = 14 nM). By the same token, the smaller ring could help [Ala7]-MST isomerize its *trans* conformation at Ala7 into the *cis* one, even if only to a small extent. This might cause [Ala7]-MST to display inhibitory activity against subtilisin, even if at two orders of magnitude less potently than the original.

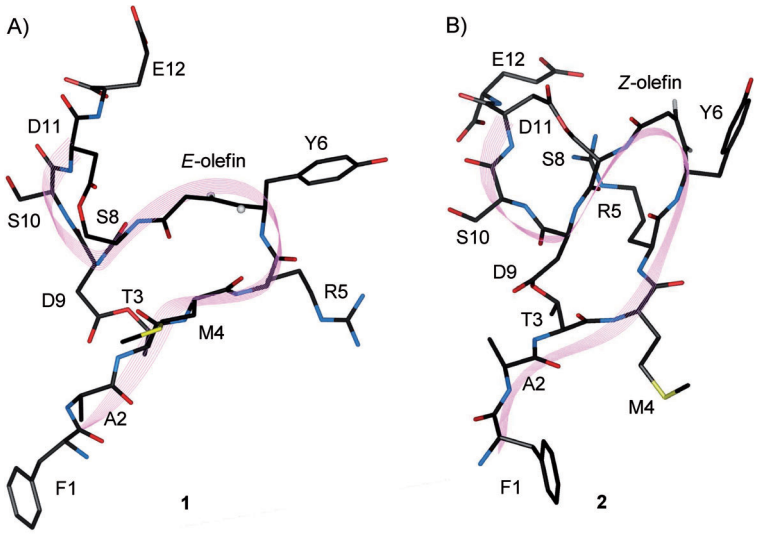



**Figure 1.** NMR solution structures of A) *E* olefin analogue **1** and B) *Z* olefin analogue **2**.

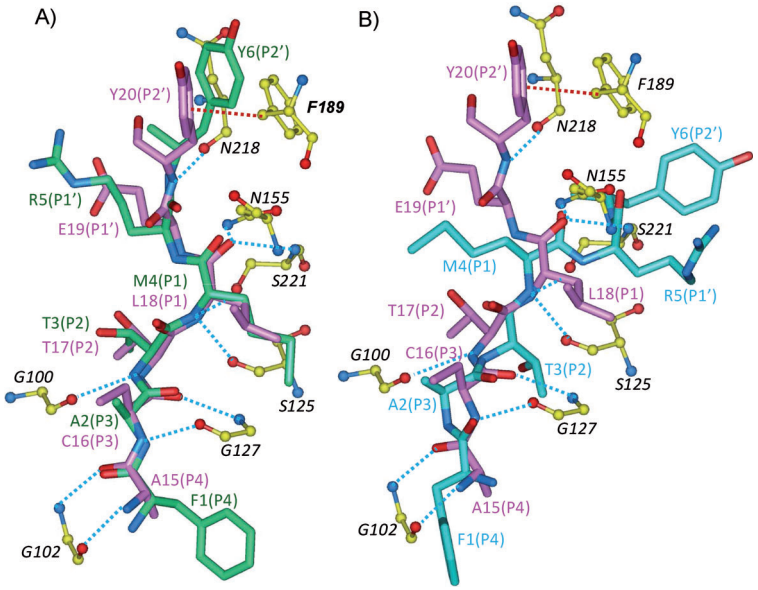



**Figure 2.** Comparison of P4–P2′ structures. A) The P4–P2′ region, Phe1–Tyr6, of the NMR solution structure of the *Z* olefin analogue **2** is superimposed on the corresponding region (Ala15–Tyr20) of OMTKY3 bound to CARL; PDB ID: 1YU6 by using the main-chain atoms. The analogue **2** and OMTKY3 are shown as stick models in green and magenta, respectively. The OMTKY3-interacting residues of CARL are shown as ball-and-stick models. Hydrogen bonds and a stacking interaction observed for the OMTKY3–CARL complex are indicated by blue and brown dotted lines, respectively. Nitrogen and oxygen atoms are shown in blue and red, respectively. B) The P4–P2′ region, Phe1–Tyr6, of the NMR solution structure of the *E* olefin analogue **1** is overlaid with the corresponding region (Ala15–Tyr20) of OMTKY3 bound to CARL; PDB ID: 1YU6. The analogue **1** and OMTKY3 are shown as stick models in blue and magenta, respectively. Nitrogen and oxygen atoms, the OMTKY3-interacting residues of CARL, hydrogen bonds and a stacking interaction observed for the OMTKY3-CARL complex are shown as in (A).

In summary, we have successfully synthesized the MST olefin analogues **1** and **2** by employing stereoselectively constructed *E* and *Z* olefin dipeptide isosteres, respectively. Analogue **1** completely lost inhibitory activity, but analogue **2** retained it. This strongly suggested that the *cis* conformation at position 7

**Table 1.** $\phi$ and $\psi$ angles of the P2–P2′ regions in MST, [Ala7]-MST, *E* olefin analogue **1**, and *Z* olefin analogue **2**.

| | | P2 (Thr) | P1(Met) | P1′(Arg) | P2′(Tyr) |
|---|---|---|---|---|---|
| [Ala7]-MST | $\phi$ | −141.9 | −159.6 | −65.1 | −49.5 |
| | $\psi$ | 133.5 | 9.8 | −81.3 | −55 |
| **1** | $\phi$ | −138.8 | −159.5 | −33.5 | −105.7 |
| | $\psi$ | 156.9 | 17.2 | −61.4 | −70.2 |
| MST | $\phi$ | −70.9 | −103.2 | −43.3 | −107.0 |
| | $\psi$ | 153.9 | 23.6 | 170.2 | 121.2 |
| **2** | $\phi$ | −59.5 | −101.5 | −47.2 | −110.9 |
| | $\psi$ | 152.8 | 26.1 | 162.0 | 112.8 |

**Table 2.** $K_i$ values of MST analogues against CARL.

| MST analogues | $K_i$ [nM][a] | Conformation of AA7 |
|---|---|---|
| MST | $0.59 \pm 0.05$[b] | *cis* |
| **2** | $6.6 \pm 1.1$ | *cis* |
| [Ala7]-MST | $84.3 \pm 1.2$[b] | *trans* |
| **1** | $>10000$ | *trans* |

[a] Means of 4–6 determinations ± SD. [b] Data from ref. [7].

plays a critical role in the MST molecule for prescribing the canonical structure bound to proteases, and that [Ala7]-MST should isomerize its *trans*-Ala7 into the *cis* one when it binds to proteases. To verify that both enzyme-bound MST and enzyme-bound [Ala7]-MST take the *cis* conformation at position 7 to express a canonical structure, analysis of their crystal structures in complex with subtilisin is now in progress.

**Keywords:** enzyme inhibitors · marinostatin · NMR spectroscopy · olefin dipeptide isosteres · structure–activity relationships

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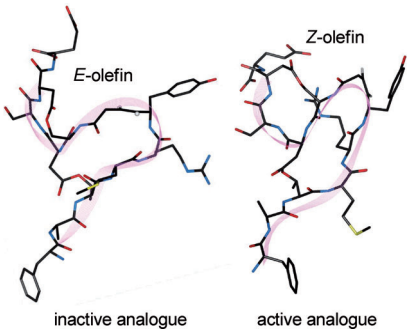
# COMMUNICATIONS

**Rational design of inhibitors:** The *cis*-amide backbone at position 7 in the serine protease inhibitor marinostatin was replaced with an *E* or *Z* olefin. The *E* olefin analogue was not active, but the *Z* analogue was. The *cis* conformation might play a critical role in organizing a canonical structure for binding to proteases.

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