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Functional Characterization of MpaG′, the O-Methyltransferase Involved in the Biosynthesis of Mycophenolic Acid

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Mycophenolic acid (MPA, **1**) is a clinically important immunosuppressant. In this report, a gene cluster *mpa′* responsible for the biosynthesis of **1** was identified from *Penicillium brevicompactum* NRRL 864. The *S*-adenosyl-L-methionine-dependent (SAM-dependent) O-methyltransferase encoded by the *mpaG′* gene was functionally and kinetically characterized in vitro. MpaG′ catalyzes the methylation of demethylmycophenolic acid (DMMPA, **6**) to form **1**. It also showed significant substrate flexibility by methylating two structural derivatives of **6** prepared by organic synthesis.

Mycophenolic acid (MPA, **1**, Scheme 1) was the first isolated antibiotic natural product in the world, obtained from a culture of *Penicillium* sp. in 1893.[1] Since then, a number of different *Penicillium* strains have been reported to have the ability to produce **1**.[2] It is known that **1** possesses a wide spectrum of bioactivities such as antiviral,[3] antifungal,[4] antibacterial,[5] antitumor,[6] and antipsoriasis activities.[7] In particular, **1** has been developed into a clinically important immunosuppressant, with its derivatives currently marketed under the brand names of CellCept (Roche) and Myfortic (Novartis).[8]

Because of its interesting polyketide/terpene hybrid structure and remarkable bioactivity, **1** has attracted extensive attention with regard to its chemical derivatization[1] and biosynthetic mechanism. Previous precursor labeling and culture feeding studies had provided preliminary insights into the biogenesis of **1**.[9] However, the biosynthetic pathway to **1** remained unclear at the genetic level until the recent discovery of the gene cluster *mpa*, responsible for the assembly of **1**, from *Penicillium brevicompactum* IBT23078.[8]

During our independent whole-genome sequencing and analysis of *P. brevicompactum* strain NRRL 864, which had previously been reported to be a producer of **1**,[10] we identified the gene cluster *mpa′* for the biosynthesis of **1** (Scheme 1, Table S1 in the Supporting Information, Genbank accession number: KM595305). This gene cluster is highly similar to *mpa*, with identical gene organization and 96.4% overall DNA sequence identity, although its upstream and downstream genes (*orf1–4*) are different from those of *mpa*. Like *mpa*, *mpa′* consists of seven open reading frames (ORFs) that putatively encode one nonreducing polyketide synthase (MpaC′), one prenyltransferase (MpaA′), one oxidative cleavage enzyme (MpaH′), one inosine-5′-monophosphate dehydrogenase (IMPDH, MpaF′), one natural fusion enzyme consisting of a cytochrome P450 domain and a hydrolase domain (MpaDE′), one O-methyltransferase (MpaG′), and one protein with unknown function (MpaB′, Table S1).

Functionally, MpaC′ (95.9% identical to MpaC[8] at the amino acid level), a nonreducing polyketide synthase containing the starter unit acyl carrier protein transacylase (SAT), β-ketoacylsynthase (KS), acyltransferase (AT), product template (PT), acyl carrier protein (ACP), and methyltransferase (MT) domains, is likely responsible for the production of 5-methylorsellinic acid (5-MOA, **2**).[11] Next, bifunctional MpaDE′, with 98.6% identity to MpaDE,[8] is predicted to mediate the methyl hydroxylation of **2** (by the P450 monooxygenase domain) to afford 4,6-dihydroxy-2-(hydroxymethyl)-3-methylbenzoic acid (DHMB, **3**) and subsequent lactonization of **3** (by the hydrolase domain) to form the phthalide intermediate 5,7-dihydroxy-4-methylphthalide (DHMP, **4**,[12] Scheme 1). MpaF′ likely confers self-resistance toward **1** by encoding the MPA-insensitive IMPDH.[13]

In view of the great similarity between *mpa* and *mpa′*, we propose that these two gene clusters should program the same biosynthetic pathway. So far, two out of seven ORFs in the *mpa* gene cluster (MpaC and MpaDE) have been characterized by gene disruption and heterologous protein expression in *Aspergillus nidulans*.[8, 11–12] However, there remain important biosynthetic steps that have yet to be functionally identified. According to bioinformatic analysis, the putative prenyltransferase MpaA′ might catalyze the farnesylation of **4** to generate

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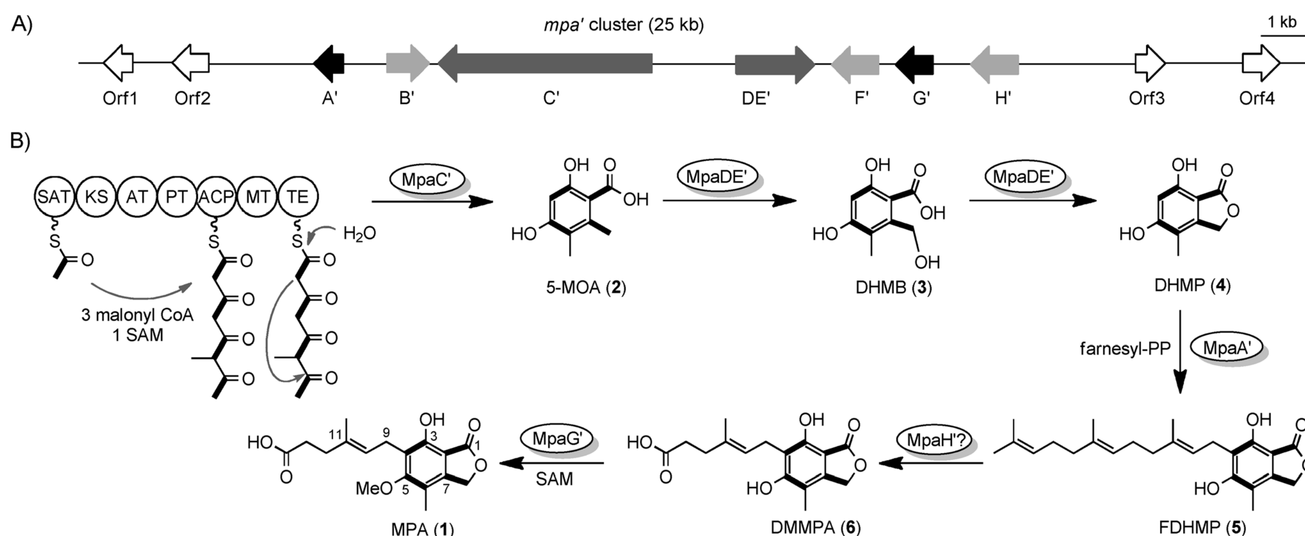


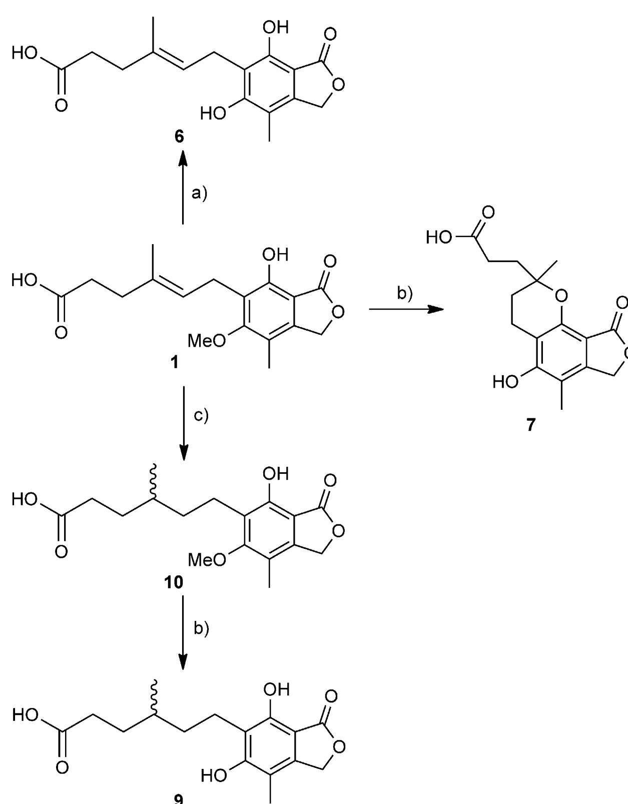
Scheme 1. A) The *mpa'* gene cluster. B) The proposed biosynthetic pathway to **1**.

6-farnesyl-5,7-dihydroxy-4-methylphthalide (FDHMP, **5**),[8] followed by the MpaH′-mediated oxidative cleavage of the farnesyl chain to give rise to demethylmycophenolic acid (DMMPA, **6**, Scheme 1);[8] **6** is likely to be converted into the final product **1** by methylation of the 5-hydroxy group by MpaG′.[8] In this study, MpaG′ was biochemically characterized in vitro.

MpaG′ consists of 398 amino acids; it encodes a putative O-methyltransferase with 99.3 % amino acid sequence identity to the previously reported MpaG (not characterized) in *P. brevicompactum* IBT23078[8] (Table S1). Protein sequence alignment of MpaG′ with its homologous proteins revealed the three conserved catalytic residues His306, Glu325, and Glu362, as well as the *S*-adenosyl-L-methionine (SAM)-binding motif (LVDVGGGxG), a signature sequence of this O-methyltransferase family[14] (Figure S1). According to the phylogenetic analysis of MpaG′ and the selected O-methyltransferases (Figure S2), MpaG′ seems to be evolutionarily related to caffeic acid O-methyltransferases.[15]

To characterize the putative function of MpaG′ biochemically, *mpaG′* was cloned into pET-28b and overexpressed in *Escherichia coli* BL21(DE3). The recombinant N-terminal His_6-tagged protein was purified to homogeneity by one-step Ni-NTA agarose chromatography. The substrate **6**, which is not commercially available, was chemically prepared from **1** (Sigma–Aldrich); the methoxy group on C5 was cleaved in the presence of lithium iodide[16] with a yield of 88.5 % (Scheme 2, for details see the Supporting Information). Compound **6** was sequentially purified by silica gel chromatography and semipreparative reversed-phase C18 HPLC. Its structure was confirmed by HRMS (ESI, $[M+H]^+$: calcd: 307.1176; found: 307.1173) and NMR analysis (Figures S3 and S4).

With SAM as the methyl donor and **6** as substrate, the activity of MpaG′ was reconstituted in vitro (sodium phosphate buffer, 50 mM, pH 8.0) at 28 °C. LC-MS analysis showed that a new peak with the same retention time and molecular weight as the authentic standard **1** was produced with consumption of **6** (Figure 1). This result confirmed that MpaG′ is responsible for the final step in the MPA biosynthetic pathway (Scheme 1).



Scheme 2. Chemical synthesis of variant substrates for MpaG′. a) LiI, collidine; b) BBr_3/CH_2Cl_2; c) Pd/C, H_2, MeOH.

However, we currently cannot exclude the possibility that MpaG′ might also use **2**, **3**, **4**, or **5** as alternative substrates, due to unavailability of these compounds. Notably, the previous substrate feeding study strongly suggested that farnesylation of **4** should occur earlier than the methylation step be-

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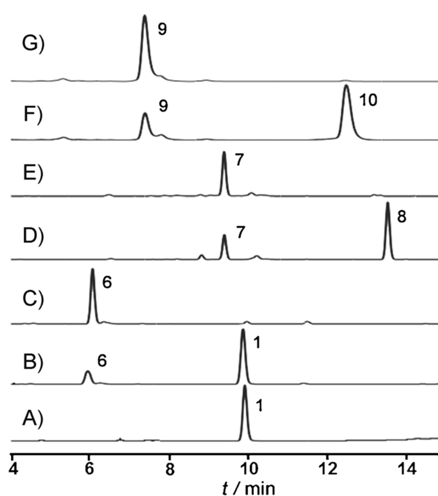


Figure 1. HPLC analysis (λ = 254 nm) of in vitro conversions catalyzed by MpaG′. A) Authentic standard of **1**, B) **6** + MpaG′ + SAM, C) **6** + MpaG′ as control, D) **7** + MpaG′ + SAM, E) **7** + MpaG′ as control; F) **9** + MpaG′ + SAM, and G) **9** + MpaG′ as control. Compound identities were confirmed by HRMS and NMR spectroscopy.

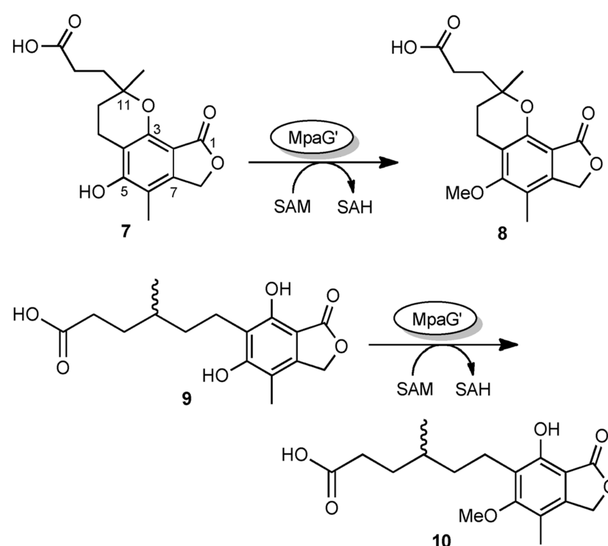
cause the incorporation of labeled **4** and its 5-O-methyl derivative gave a 10:1 product ratio during the production of **1**.[9c]

To investigate the catalytic mechanism of MpaG′, we mutated the three putative catalytic residues His306, Glu325, and Glu362 to alanine. According to previous mechanistic studies,[14] His306 is likely responsible for deprotonating the C5-OH group to generate a hydroxy anion, which attacks the methyl group of SAM. The two glutamate residues might interact with His_{306} through hydrogen bonds to ensure the optimal position and orientation of the imidazole group for deprotonation. As a result, the purified MpaG′ mutants H306A, E325A, and E362A (Figure S5) completely lost any methyltransferase activity towards **6** (Figure S6), thus confirming the critical functionality of these amino acids.

The activity of MpaG′ is not dependent on addition of divalent metal ions (data not shown). This is in good agreement with the previous report.[9b] The optimal pH and temperature for MpaG′ were determined through comparison of enzymatic activities under variant reaction conditions. The results showed that MpaG′ displayed the highest activity at pH 8.0 (Figure S7), similar to the previously reported optimal pH of an O-methyltransferase isolated from *Penicillium stoloniferum* AAO-05115 that is also involved in MPA biosynthesis.[9b] At pH 8.0, the maximum activity of MpaG′ was observed at 45 °C (Figure S7), higher than that of the O-methyltransferase from the AAO-05115 strain.[9b]

Under the optimal conditions, the steady-state kinetic parameters of MpaG′ were determined on the basis of consumption of **6**, monitored by HPLC (Figure S8). MpaG′ converted **6** into **1** with an apparent K_M value of 51.5 ± 13.0 μm and a k_{cat} value of 26.2 ± 2.4 min^{-1}. In relation to the previously reported kinetic data for the O-methyltransferase (with no sequence information) isolated from another MPA-producing strain (*P. stoloniferum* AAO-05115), MpaG′ methylates **6** approximately two times more efficiently with respect to k_{cat}/K_M value.[9b]

During our chemical modification of **1** by use of boron tribromide as the demethylation reagent in an acidic environment, we obtained cyclized product **7** (Scheme 2, Figures S9 and S10), with a molecular weight ([M+H]$^+$: calcd.: 307.1176; found: 307.1174) identical to that of **6**, according to HRMS (ESI) analysis. Despite the significant structural difference between **6** and **7**, we were surprised to observe that MpaG′ was able to methylate **7** in the presence of SAM under the optimal conditions to afford the new methylated product **8** (Figure 1 and Scheme 3). This result demonstrates the substrate flexibility of MpaG′.



Scheme 3. Unnatural reactions catalyzed by MpaG′.

It was reported that under strongly acidic conditions, three isomers could be expected: two chromans formed between C11 and the two different hydroxy groups on the aromatic ring, or the lactone between C11 and the terminal carboxylic acid group (Figure S11).[17] Simple 1D and 2D NMR experiments had difficulty in distinguishing these three structures, because they would display similar proton coupling patterns. We thus performed the MpaG′ enzymatic reaction with **7** as substrate on a preparative scale and purified the methylated product as compound **8** (Figures S12 and S13). The structure of **8** was elucidated by 1D and 2D NMR. HMBC correlation analysis clearly showed a strong signal between the newly installed methyl group (δ_H = 3.79 ppm) and C5 (δ_C = 163.6 ppm) of **8** (Figure S13); this is the same methylation position as when **6** was the substrate. Therefore, compound **7** was deduced to be 2,11-*cyclo*-DMMPA. In addition, HPLC analysis showed that **1** could not be further methylated when it was incubated with MpaG′ (Figure S14). Taken together, these results suggest that the C5 hydroxy group is essential for substrate recognition by MpaG′, whereas the C3 hydroxy group is inert to MpaG′.

The unexpected substrate tolerance of MpaG′ prompted us to test whether the analogous structure **9** (10,11-saturated DMMPA) could be another potential substrate. To obtain **9**, the C10=C11 double bond of **1** was first reduced in the presence

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of palladium (10% on carbon) to afford the racemic intermediate **10** (Scheme 2, Figures S15 and S16, Supporting Information), and the C5 methyl group of **10** was subsequently cleaved by treatment with boron tribromide under acidic conditions to afford **9** (Figures S17 and S18). After 1 h incubation of **9** with MpaG′ and SAM, **9** was successfully converted into the methylated product as **10** (Figure 1 and Scheme 3); this was supported by HRMS (ESI, $[M+H]^+$: calcd: 323.1489; found: 323.1487).

In summary, we identified the second mycophenolic acid biosynthetic gene cluster (*mpa′*) by sequencing and mining the whole genome of *P. brevicompactum* NRRL 864. The O-methyltransferase MpaG′, catalyzing the final step of MPA biosynthesis, was functionally characterized in vitro. Although a functionally similar protein had been obtained from *P. stoloniferum* AAO-051,[12] it was only partially characterized as an O-methyltransferase responsible for the C5 O-methylation of **6** without the DNA sequence being obtained, due to technical limitations at that time. Our present study provides detailed biochemical information on this important tailoring enzyme for the first time. The mutagenesis analysis of MpaG′ confirmed the catalytic triad of His306-Glu325-Glu362. The discovery of the unusual substrate flexibility of MpaG′ could be useful for enzymatic generation of new MPA derivatives for future drug development. The structural elucidation of MpaG′ should shed light on understanding of its broad substrate specificity, and this is currently under investigation in our laboratories.

Experimental Section

General experimental procedures: Liquid chromatography mass spectrometry (LC-MS) analysis was carried out with an Agilent 1260–6430 spectrometer and a Waters symmetry column (4.6× 150 mm, RP18) with a water (0.1% formic acid) and acetonitrile (0.1% formic acid) biphasic solvent system. HRMS were recorded with a Dionex Ultimate 3000 instrument coupled to a Bruker Maxis Q-TOF spectrometer. NMR spectra were acquired with a Bruker 600 MHz spectrometer. NMR data were processed by use of Topspin software. Reversed-phase HPLC (RP-HPLC) was employed to purify MPA derivatives with use of a Waters X-Bridge 5 μm C18 column and a solvent system of acetonitrile and water supplemented with trifluoroacetic acid (TFA, 0.1%). The UV/Vis spectra were taken with a Spectrophotometer DU800 (Beckman Coulter). Primer synthesis and DNA sequencing was performed by Sangon Biotech, Shanghai, China.

MpaG′ gene cloning: The 7 day mycelia of *Penicillium brevicompactum* NRRL 864 were ground to powder in liquid nitrogen. TRIZOL solution (Life Sciences, 1 mL) was added to the mycelia powder (100 mg) and mixed well. Total RNA was extracted according to the manufacturer's manual (Life Sciences). Reverse transcription PCR (RT-PCR) was performed with a kit purchased from Takara according to the protocol supplied by the manufacturer (6110A, Takara Bio, Inc.). The gene encoding MpaG′ was PCR-amplified by use of cDNA as template and the primer pair of MpaG′_NdeI: GGAAT TCCAT ATGAG TGCCG CATCC CCAG and MpaG′_BamHI: CGGGA TCCGG CGAGT TCCGC TTCAA TC. The gel-cleaned PCR fragment was double digested with NdeI and BamHI and subsequently inserted into the NdeI/BamHI pretreated pET-28b to afford pET28b-*mpaG′*.

Protein expression and purification: Recombinant plasmids pET28b-mpaG′ were used to transform *E. coli* BL21(DE3) cells. The resulting transformants were grown at 37 °C in lysogeny broth (1 L) containing kanamycin (50 mg mL^{-1}) for 2–3 h until OD_{600} reached 0.4–0.6. Isopropyl β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.2 mM to induce gene expression, and the cells were cultured at 18 °C overnight. The culture was centrifuged at 6000 *g* for 10 min to collect cells. The freeze-thaw cell pellet was resuspended in lysis buffer [30 mL, NaH_2PO_4 (50 mM), NaCl (300 mM), imidazole (10 mM), glycerol (10%), pH 8.0] and applied to sonication. Cell debris was removed by centrifugation at 10000 *g* for 60 min, and the supernatant was mixed with Ni-NTA agarose (Qiagen, 1 mL) for 1 h at 4 °C. The slurry was loaded onto an empty column, and the column was washed stepwise with lysis buffer (10 mL) and wash buffer [40–60 mL, NaH_2PO_4 (50 mM), NaCl (300 mM), imidazole (20 mM), glycerol (10%), pH 8.0]. The bound His_6-tagged proteins were eluted with elution buffer [NaH_2PO_4 (50 mM), NaCl (300 mM), imidazole (250 mM), glycerol (10%), pH 8.0]. The MpaG′ (≈43 kDa) proteins were further purified and concentrated by use of a 30 kDa size-exclusion filter (Amicon). The final desalting step was achieved by buffer exchange into storage buffer [NaH_2PO_4 (50 mM), glycerol (10%), pH 7.4] with a PD-10 column (GE Healthcare). The concentration of MpaG′ was determined by Bradford assay with bovine serum albumin (BSA) as standard.

Site-directed mutagenesis: The three MpaG′ mutants H306A, E325A, and E362A were generated by site-directed mutagenesis by use of the QuikChange Kit from Stratagene according to the manufacturer's instructions. The plasmid pET28b-*mpaG′* was employed as template. The mutagenic primers used to introduce different mutations are listed in Table S2. The mutated sequences were confirmed by DNA sequencing, and transformed into the competent cells of *E. coli* BL21(DE3). The mutant proteins were expressed and purified as described above.

MpaG′ enzymatic assay: The standard assay containing wild-type or mutant MpaG′ (1 μM), substrate (0.5 mM), and SAM (5.0 mM) in reaction buffer [100 μL, NaH_2PO_4 (50 mM), pH 8.0, glycerol (10%)] was carried out at 28 °C for 1 h and quenched with an equal volume of chloroform. The organic extract was dried under N_2 flow and redissolved in methanol (100 μL) for HPLC and LC-MS analysis.

Steady-state kinetics: The standard reaction in the reaction buffer [NaH_2PO_4 (50 mM), pH 8.0, glycerol (10%)] contained MpaG′ (10–100 nM) and DMMPA (10–500 μM) in a total volume of 142.5 μL. After preincubation at 45 °C for 5 min, the reactions with different substrate concentrations were initiated by adding SAM (7.5 μL) to the final concentration of 500 μM, and three aliquots (50 μL) were taken at three time points (0, 0.5, 1 min and 0, 1.5, 2 min for reactions with substrate concentrations <50 μM and >100 μM, respectively) within the linear range to mix thoroughly with methanol (50 μL) for reaction termination. The proteins were removed by centrifugation at 16000 *g* for 15 min. The supernatant was subject to HPLC analysis to monitor substrate consumption within the linear range, thereby allowing calculation of the initial velocity of O-methylation reactions. The HPLC conditions were as follows: reversed-phase HPLC column (C18, 5 μm, 150 mm, Thermo), 40–60% solvent B over 20 min (solvent A = deionized H_2O + 0.1% TFA; solvent B = acetonitrile + 0.1% TFA), flow rate 1.0 mL min^{-1}, UV wavelength 254 nm. All measurements were performed in duplicate, and velocities determined at different substrate concentrations were fit into the Michaelis–Menten equation to calculate the kinetic parameters.

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