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Synthesis and Antiaggregative Activity of a New RGDF Mimetic

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Abstract—*m*-[4-Oxo-4-(piperazin-1-yl)butyrylamino)benzoyl-*D*,*L*-β-(3,4-methylenedioxyphenyl)-β-alanine, a new mimetic of the peptide RGDF, was synthesized. This compound inhibited ADP-induced platelet aggregation in human blood plasma enriched with platelets with $IC_{50} = 3.5 \times 10^{-8}$ M.

Key words: GP IIb/IIIa, peptide synthesis, platelet aggregation, RGDF mimetic

INTRODUCTION

A search for new antithrombotic and antiaggregative agents with high efficiency and low toxicity is one of the most important problems of bioorganic and medicinal chemistry.[2]

In the last ten years, sedulous attention has been paid to a group of antiaggregative agents that are antagonists of the GP IIb/IIIa fibrinogen receptors. The tripeptide sequence RGD (Arg-Gly-Asp) is responsible for the binding of fibrinogen to its receptors. Small proteins and peptides containing the RGD sequence are antagonists of the fibrinogen receptors, and the peptides involving this sequence can be used as inhibitors of the platelet aggregation. The molecular design of potential antagonists of the fibrinogen receptors is aimed at an increase in their selectivity and affinity and involves the modeling of structures containing this sequence or fragments mimicking it.

The molecules of highly active RGDF mimetics should have a certain distance (10–15 Å) between two binding centers: the terminal basic group (guanidino, amidino, amino, or another group) and carboxyl function [1]. The structure of peptidomimetic (**II**), which was prepared by Alig *et al.* [2] by a modification of cyclopeptide (**I**) (Scheme 1), meets these requirements. Introduction of the residue of *m*-aminobenzoic acid provides for the substantial rigidity of the mimetic molecule and exerts a positive effect on its activity. The IC_{50} value for the antiaggregative activity of (**II**) is 200 nM [2].

Previously, we synthesized a number of linear RGDF peptidomimetics (**III**) and (**IV**) on the basis of 4-oxo-4-(piperazin-1-yl)butyric acid mimicking the Arg residue. The Asp-Phe sequence was replaced by the residues of β-substituted β-alanines [3–5]. These compounds exhibited sufficiently high antiaggregative activities (IC_{50} varied from 10^{-6} to 10^{-8} M). Affinities of the most active compounds to the fibrinogen receptors was studied by the method of fluorescent analysis [4, 5].

RESULTS AND DISCUSSION

The goal of this study was the synthesis of a new RGDF peptidomimetic (**XII**) that contains the residue of 4-oxo-(4-piperazin-1-yl)butyric acid instead of Arg, a fragment of *m*-aminobenzoic acid, and the residue of *D*,*L*-β-(3,4-methylenedioxyphenyl)-β-alanine. The *C*-terminal hydrophobic radical is necessary for the effective binding of the mimetic to the receptor [6].

The synthesis of pseudopeptide (**XII**) is presented in scheme 1. 4-Oxo-4-(4-Boc-piperazin-1-yl)butyric acid (**V**) was used as a starting compound [3]. The condensation of *m*-aminobenzoic acid and Boc-derivative (**V**) was carried out by three methods. The first method consisted in the use of mixed anhydride (**VIII**), which was prepared from the triethylammonium salt of acid (**V**) and isobutyl chloroformate. Succinimide ester (**VI**) and pentafluorophenyl ester (**VII**) were used in the second and in the third methods, respectively. The esters (**VI**) and (**VII**) were synthesized using DCC and *N*-hydroxysuccinimide or pentafluorophenol, respectively. The amino group of *m*-aminobenzoic acid was acylated with derivatives (**VI**)–(**VIII**). The best result was achieved with pentafluorophenyl ester (**VII**); an average yield of Boc-derivative (**IX**) was approximately 80%. The next stage was an activation of carboxyl group in (**IX**) by DCC in the presence of HONSu. The

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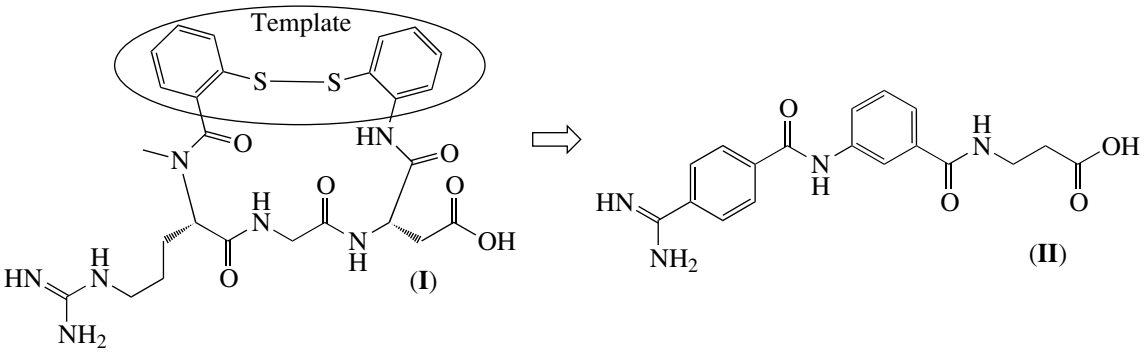
[2] Abbreviations: β-Ala, β-alanine, DCC, *N*,*N*'-dicyclohexylcarbodiimide; FAB, fast atom bombardment; HONSu, *N*-hydroxysuccinimide; PfpOH, pentafluorophenol; Piz, piperazinyl; and Suc, succinyl.



Scheme 1. The conversion of cyclopeptide (**I**) into the RGDF mimetic (**II**).

corresponding succinimide ester (**X**) resulted. It was reacted with the sodium salt of *D,L*-β-(3,4-methylenedioxyphenyl)-β-alanine [7], which resulted in Boc-derivative (**XI**). The target (**XII**) was prepared by the acidolytic removal of Boc-group from (**XI**).

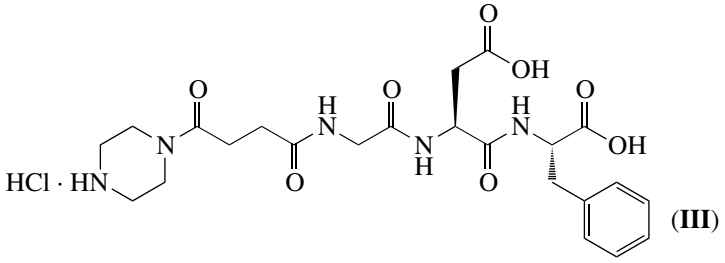
Structures of the synthesized compounds were confirmed by mass spectrometry and ^{1}H NMR spectroscopy. All the characteristic signals from protons with the corresponding integral intensities were present in the ^{1}H NMR spectra of the synthesized compounds.

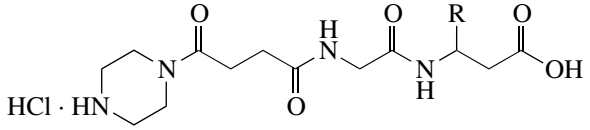
The antiaggregative activity of the prepared RGDF mimetic (**XII**) was studied on human blood plasma rich with platelets according to the Born method [8]. Compound (**XII**) inhibited the ADP-induced aggregation of platelets with IC_{50} of 3.5×10^{-8} M. We should note that the IC_{50} value for the RGDF peptide was 1.9×10^{-6} M. Thus, the RGDF mimetic (**XII**) exhibited a high antiaggregative activity.

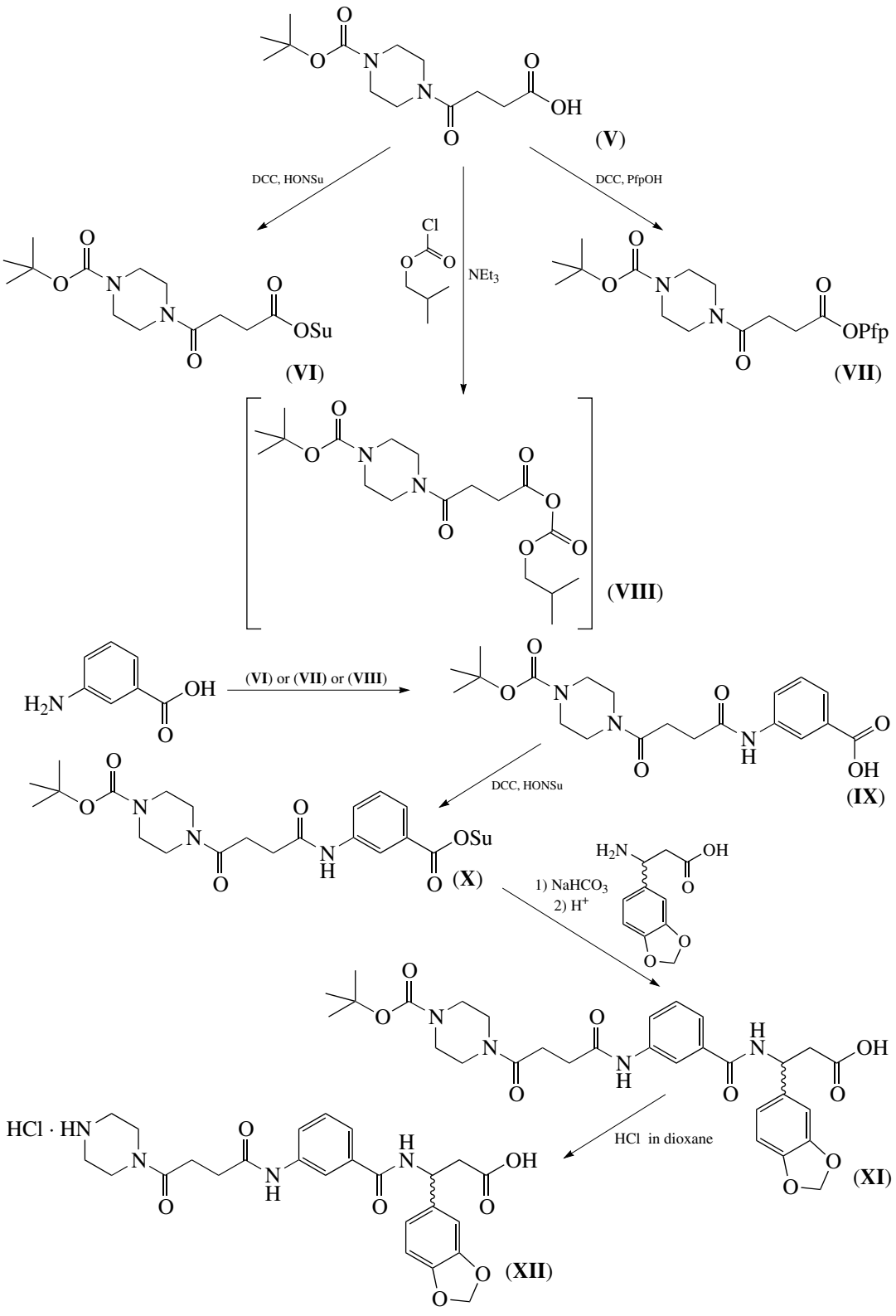
EXPERIMENTAL

The ^{1}H NMR spectra were recorded on a Varian WXP-300 spectrometer (Varian, Germany) with a working frequency of 299.95 MHz in DMSO-d_6 (99.9%) at the temperature of 25°C. Tetramethylsilane was used as an internal standard. The mass spectrum of (**VI**) was recorded on an MX-1321 mass spectrometer (NPO Nauchnyi Pribor, Russia) at the ionization energy of 70 eV and at the temperature of 200°C in the ionization chamber. The FAB mass spectra were recorded on a VG 7070 spectrometer (VG, UK) using a glycerol matrix with the ionization by a beam of Xe atoms with the energy of 8 kV. The purity of compounds was examined by HPLC on a DuPont 8800 chromatograph (DuPont Instruments, United States) equipped with a Zorbax C8 analytical column (DuPont Instruments, United States) eluted with a 4 : 1 acetonitrile–water mixture. TLC was carried out on Silufol sheets (Kavalier, Czech Republic) and Kieselgel 60 precoated plates (Merck, Germany) in the following chromatographic systems: (A) 100 : 50 : 1 benzene–acetone–acetic acid, (B) 4 : 1 methanol–ammonia, (C) 9 : 3 : 2 chloroform–ethyl acetate–methanol, and (D) 9 : 3 : 2 : 1 chloroform–ethyl acetate–methanol–acetic acid. The substances were detected by ninhydrin or by chlorine–toluidine reagent.

The following designations are used in the description of ^{1}H NMR spectra: Ar-βAla, aromatic protons of the β-(3,4-methylenedioxyphenyl)-β-alanine residue; $^{\alpha}CH_2$-β-Ala, α-protons of the β-aryl-substituted β-alanine residue; $^{\beta}$CH-β-Ala, β-proton of the residue of the







Scheme 2. The synthesis of *m*-[4-oxo-4-(piperazin-1-yl)butyrylamino]benzoyl-*DL*-β-(3,4-methylenedioxyphenyl)-β-alanine (**XII**).

aryl-substituted β-alanine; CH_2-NSu, protons of the succinyl residue; CH_2-Piz, protons of the piperazinyl residue; and NH-β-Ala, amide proton of the residue of β-substituted β-alanine.

Succinimidyl 4-oxo-4-(4-Boc-piperazin-1-yl)butyrate (VI). HONSu (2.414 g, 20.9 mmol) and DCC (4.329 g, 20.9 mmol) were added to a solution of (**V**) (5 g, 17.5 mmol) [3] in anhydrous THF (40 ml). The reaction mixture was cooled to 0°C and stirred at this temperature for 4 h. When the reaction was completed, the precipitated *N*,*N*'-dicyclohexylurea was filtered off, and the filtrate was evaporated in a vacuum at the temperature no higher than 35°C. The residue was recrystallized from isopropanol, washed with cool isopropanol and ether, and dried in desiccator over $CaCl_2$ to give (**VI**); yield 5.825 g (87%); R_f 0.41 (A), 0.56 (D); mp 155–159°C; mass spectrum, *m*/*z* (*I*, %): 383 (5.1), 327 (6.4), 310 (12.7), 269 (22.4), 213 (40.6), 212 (14.4), 211 (5.2), 168 (6.0), 167 (5.8), 115 (9.3), 100 (11.5), 97 (12.5), 86 (5.0), 84 (20.9), 83 (6.8), 69 (16.4), 58 (6.1), 57 (100), 56 (33.8), 55 (45.1); ^{1}H NMR (δ, ppm): 1.41 (9 H, s, CH_3-Boc), 2.70 (2 H, t, CH_2-NSuc), 2.79 (4 H, s, CH_2-NSu) 2.87 (2 H, t, CH_2-Suc), 3.28–3.34 (4 H, m, CH_2-Piz), and 3.42–3.4 (4 H, m, CH_2-Piz).

***m*-[4-Oxo-4-(4-Boc-piperazin-1-yl)butyrylamino]-benzoic acid (IX). Method A.** Triethylamine (0.58 ml, 4.2 mmol) was added to a solution of (**V**) (1 g, 3.5 mmol) in chloroform (15 ml). The solution was cooled to –5°C, and isobutyl chloroformate (0.5 ml, 3.8 mmol) was added. The reaction mixture was kept for 1 h at –5°C, and *m*-aminobenzoic acid (0.578 g, 4.2 mmol) was added. The reaction mixture was refluxed for 3 h, washed with 1 M solution of HCl (2 × 10 ml), water (2 × 10 ml), and a saturated solution of NaCl (10 ml), and dried with anhydrous sodium sulfate. Sodium sulfate was filtered off, chloroform was evaporated, and the residue was dried in a vacuum. Ether was added to the oily residue, and the mixture was triturated to crystallization of the product. The crystals were filtered, washed with ether, and dried. The yield 0.76 g (35%).

Method B. *m*-Aminobenzoic acid (4.29 g, 31 mmol) was added to a solution of (**VI**) (10 g, 26mmol) in THF (40 ml). The reaction mixture was refluxed for 3 h and evaporated under a reduced pressure. Water (50 ml) was added to the residue, and the mixture was acidified to pH 3–4 with concentrated HCl and extracted with chloroform. The chloroform extract was washed with 1 M HCl (2 × 50 ml), water (2 × 50 ml), and a saturated solution of NaCl (50 ml); dried with anhydrous sodium sulfate; and evaporated. The residue was dried in a vacuum, mixed with ether, and triturated to the crystallization of oily residue. The crystals were filtered, washed with ether, and dried. The yield was 6.66 g (63%).

Method C. This method is based on the reaction of pentafluorofenyl ester (**VII**) [3] with *m*-aminobenzoic acid. Preparation of the product and its isolation were carried out as described in the method B. The yield 87%; R_f 0.39 (C), 0.45 (D); mp 155°C; FAB MS, *m*/*z*: 406 [*M* + H]$^+$; ^{1}H NMR (δ, ppm): 1.41 (9 H, s, CH_3-Boc), 2.63 (4 H, dd, CH_2-Suc), 3.28–3.36 (4 H, m, CH_2-Piz), 3.42–3.46 (4 H, m, CH_2-Piz), 7.41 (1 H, t, Ar), 7.59 (1 H, d, Ar), 7.80 (1 H, d, Ar), 8.24 (1 H, s, Ar), 10.14 (1 H, s, NH), and 12.92 (1 H, br. s, COOH).

Succinimidyl *m*-[4-oxo-4-(4-Boc-piperazin-1-yl)butyrylamino]benzoate (X). HONSu (2.414 g, 20.9 mmol) and DCC (4.329 g, 20.9 mmol) were added to a solution of (**IX**) (7.087 g, 17.5 mmol) in anhydrous THF (30 ml). The reaction mixture was cooled to 0°C and stirred at this temperature for 4 h. The precipitated *N*,*N*'-dicyclohexylurea was filtered off, and the filtrate was evaporated at the temperature no higher than 35°C. The residue was recrystallized from isopropanol. The precipitated product was washed with cool isopropanol and ether and dried in a desiccator over $CaCl_2$ to give (**X**); yield 7.028 g (80%); R_f 0.52 (C); mp 185–189°C; FAB MS, *m*/*z*: 503 [*M* + H]$^+$; ^{1}H NMR (δ, ppm): 1.41 (9 H, s, CH_3-Boc), 2.63 (4 H, dd, CH_2-Suc), 2.79 (4 H, s, CH_2-NSu), 3.29–3.36 (4 H, m, CH_2-Piz), 3.42–3.46 (4 H, m, CH_2-Piz), 7.58 (1 H, t, Ar), 7.74 (1 H, d, Ar), 7.91 (1 H, d, Ar), 8.49 (1 H, s, Ar), 10.33 (1 H, s, NH).

***m*-[4-Oxo-4-(4-Boc-piperazin-1-yl)butyrylamino]benzoyl-*DL*-β-(3,4-methylenedioxyphenyl)-β-alanine (XI).** A solution of β-(3,4-methylenedioxyphenyl)-β-alanine (1.421 g, 8.2 mmol) [7] and $NaHCO_3$ (0.687 g, 8.2 mmol) in water (20 ml) was added to a solution of (**X**) (3.413 g, 6.8 mmol) in a freshly distilled THF (20 ml). When the reaction was over, the reaction mixture was evaporated in a vacuum to the half of its initial volume, acidified to pH 4 with 3 N HCl, and extracted with chloroform (3 × 50 ml). The organic layer was washed with 1 N HCl (10 ml) and water (10 ml), dried with anhydrous Na_2SO_4, and evaporated in a vacuum. The oily residue was triturated with hot ether for the further purification, cooled, and filtered to give (**XI**); yield 3.85 g (95%); R_f 0.47 (C) and 0.58 (D); mp 160°C; FAB MS, *m*/*z*: 619 [*M* + Na]$^+$, 641 [*M* – H + 2Na]$^+$; ^{1}H NMR (δ, ppm): 1.41 (9 H, s, CH_3-Boc), 2.60 (4 H, dd, CH_2-Suc), 2.80 (2 H, ddd, $^{\alpha}CH_2$-βAla), 3.29–3.45 (8 H, m, CH_2-Piz), 5.34 (1 H, q, $^{\beta}CH$-βAla), 5.97 (2 H, s, OCH_2O), 6.82–6.88 (2 H, m, Ar-βAla), 7.00 (1 H, s, Ar-βAla), 7.36 (1 H, t, Ar), 7.47 (1 H, d, Ar), 7.77 (1 H, d, Ar), 7.95 (1 H, s, Ar), 8.74 (1 H, d, NH-βAla), 10.06 (1 H, s, NH-Ar), and 12.21 (1 H, br. s, COOH).

***m*-[4-Oxo-4-(piperazin-1-yl)butyrylamino]benzoyl-*DL*-β-(3,4-methylenedioxyphenyl)-β-alanine hydrochloride (XII).** A suspension of Boc-derivative (**XI**) (0.5 g, 1 mmol) in 4 M solution of HCl in dioxane (10 ml) was stirred for 1 h at room temperature and evaporated. The solid residue was dried for 5 h at 40°C and at the pressure of 3 mmHg. The substance (**XII**) is hygroscopic; yield 0.536 g (quantitative); R_f 0.6 (B);

FAB MS, *m*/*z*: 497 [*m*/*z*: 497 $[M + H]^+$, 519 $[M + Na]^+$, 541 $[M - H + 2Na]^+$; ^1H NMR (δ, ppm): 2.62 (4 H, dd, CH_2-Suc), 2.70–2.90 (2 H, m, $^{\alpha}CH_2$-βAla), 3.49 (4 H, d, CH_2-Piz), 3.71 (4 H, d, CH_2-Piz), 5.33 (1 H, q, $^{\beta}CH$-βAla), 5.98 (2 H, s, OCH_2O), 6.85 (2 H, s, Ar-βAla), 7.01 (1 H, s, Ar-βAla), 7.37 (1 H, t, Ar), 7.49 (1 H, d, Ar), 7.76 (1 H, d, Ar), 7.97 (1 H, s, Ar), 8.79 (1 H, d, NH-βAla), 9.25 (2 H, s, H_2N^+), 10.16 (1 H, s, NH-Ar), and 12.41 (1 H, br. s, COOH).

Pharmacological part. The platelet aggregation was studied on the platelet rich plasma (PRP) [8] prepared by centrifugation of citrate human blood at 200 *g* for 15 min. The aggregation of platelets was measured according to increase in the light transparency on a Thromlite 1006A aggregometer (Biokhimmak, Moscow, Russia) connected with a recording device. PRPs (250 μl) were incubated with the studied compound (**XII**) taken in various concentrations at 37°C for 1 min before the addition of ADP (the final concentration was 10 μM). The platelet aggregation was measured for 2 min (maximum aggregation). The biological activity of RGDF mimetic (**XII**) was quantitatively characterized by the IC_{50} value, the substance concentration at which the maximum amplitude of the platelet aggregation is 50% from the starting level.

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