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Full Length Research Paper

Design, synthesis and antibacterial activity of a novel hybrid antimicrobial peptide LFM23

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Antimicrobial peptides produced by many tissues and cell types of invertebrates, insects and humans as part of their innate immune system, have received increasing attention as potential candidates due to their administration as pharmaceutical agents. In the present study, a novel hybrid antimicrobial peptide LFM23 consisting of 23 amino acid residues was designed based on the primary sequences of bovine lactoferricin (LfcinB) and melittin. The peptide was synthesized by chemical method of solid-phase synthesis with a purity of more than 98% after reverse phase high performance liquid chromatography. Antimicrobial activity assay showed that LFM23 had strong antibacterial abilities, and the minimum inhibitory concentrations against *Escherichia coli* ATCC25922, *Salmonella typhimurium* ATCC12291, *Pseudomonas aeruginosa* ATCC27853, *Staphylococcus aureus* ATCC25923, *Pichia pastoris* GS115, were 32, 32, 64, 32 and 256 µg/ml, respectively. The hemolytic assays indicated that LFM23 had no hemolytic action *in vitro* at antimicrobial concentration. The results demonstrate that the peptide LFM23 has a good application prospect as clinically useful antimicrobial agents.

Key words: Antimicrobial peptides, design, LfcinB, melittin, antibacterial activity.

INTRODUCTION

Antimicrobial peptides (AMPs) are small molecular effector proteins widely distributed in natural organisms (Reddy et al., 2004). AMPs are important and effective components in innate host defense system against infectious pathogens (Cederlund and Gudmundsson, 2011). They vary in primary and secondary structure, but have some common properties. Natural AMPs are cationic peptides, usually consisting of less than 50 amino acid residues, and containing four or more than four positively charged amino acids with amphiphilic properties (Baltzer and Brown, 2011; Blazyk et al., 2001). AMPs have a variety of interesting biological activities including antibacterial, antifungal, antiparasitic, antitumor and antiviral (Hancock and Diamond, 2000). In addition, most AMPs have thermal stability and even can maintain their activities when heated at 100°C for 10 to 15 min (Hancock and Chapple, 1999). Most AMPs do not target specific receptors of the pathogens but interact and permeabilize cellular membranes depending on the electrostatic interaction (Hancock and Chapple, 1999). Recently, AMPs have been extensively studied and tapped as a potential alternative to combat the increasing emergence of drug-resistant bacteria.

Bovine lactoferricin (LfcinB) was first isolated from the hydrolysate products of bovine lactoferrin after proteolytic digestion (Bellamy et al., 1992). The antibacterial activity of LfcinB is 400 times stronger than bovine lactoferrin. LfcinB is derived from 17 to 41 of amino acids of bovine lactoferrin and consists of 25 amino acids with the molecular structure of amphiphilic β-sheet. LfcinB has broad host protection properties such as antibacterial (Bellamy et al., 1992), antifungal (Wakabayashi et al., 2000) antiparasitic, antiviral (Anderson et al., 2003; Mccann et

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al., 2003), tumor inhibition (Freiburghaus et al., 2009) and immunomodulatory (Haversen et al., 2002).

Melittin is a cationic and amphipathic AMP isolated from the venom of *Apis mellifera* (honey bee) and comprising about 50% of dry weight of bee venom. It is a 26-amino acid peptide with a C-terminal amide. The amino acid sequence of melittin is GIGAVLKVLTTGLPALIS WIKRKRQQ-NH2 (Habermann and Jentsch, 1967). Melittin possesses a broad biological activity spectrum like other AMPs (Bellinghausen et al., 1997; Kubo et al., 1999; Lazarev et al., 2005).

It shows a strong amphipathic α-helix forming tendency, with a basic hydrophilic highly charged C-terminal segment. Unfortunately, melittin has strong lytic activity toward eukaryotic cells (Klocek et al., 2009), which limits its clinical use as therapeutic agents. Furthermore, recent efforts were carried out toward the synthesis of hybrid peptides combining portions of the amino acid sequences of two AMPs with different properties. A series of hybrid peptides derived from melittin combined with cecropin A or maginin were designed and synthesized. These peptides have been demonstrated to have higher antimicrobial activity and also eliminated the melittin toxicity successfully (Ferre et al., 2009; Saugar et al., 2006; Xu et al., 2007).

In the present study, a novel hybrid AMP, LFM23, consisting of 23 amino acid residues was designed based on the primary sequences of LfcinB and melittin, and synthesized by chemical method of solid-phase synthesis. The antibacterial activity of LFM23 was determined *in vitro* and the hemolysis activity was evaluated.

MATERIALS AND METHODS

Strains and erythrocyte

The following strains: *Escherichia coli* ATCC25922, *Salmonella typhimurium* ATCC12291, *Pseudomonas aeruginosa* ATCC27853, *Staphylococcus aureus* ATCC25923 and *Pichia pastoris* GS115, used to evaluate the antibacterial activity of LFM23 were obtained from the Heilongjiang Type Culture Collection (Harbin, China). Human blood used to prepare erythrocyte was kindly provided by the laboratory department of the Second Affiliated Hospital of Harbin Medical University (Harbin, China).

Design, synthesis and purification of LFM23

The peptide LFM23 was designed according to the sequences of LfcinB and melittin. LFM23 incorporated 1 to 15 amino acid residues of LfcinB, in which methionine (Met) at the position 10 was substituted with tryptophan (Trp), and 5 to 12 amino acid residues of melittin. Its molecular characterization including molecular weight, theoretical isoelectric point and net charge was calculated by the biological software ProtParam (http://us.expasy.org/tools/prot-param.html), while its secondary structure was predicted by GOR I method of Antheprot 5.0.

LFM23 was prepared by 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase chemistry as previous work (Tang and Deber, 2002, 2004). Wang resins, with the first N-Fmoc-protected amino acids already attached, were included inside the T-bags. Dicyclohexylcarbodiimide (DCC) and N-hydroxybenzotriazole (HOBt) were used as coupling reagent.

After completing the sequence, cleavage from the resin and simultaneous side-chain deprotection was achieved with a mixture of trifluoroacetic acid/water/phenol/thioanisole/ethanedithiol (82.5:5:5:5:2.5, v/v) for 2 h at room temperature.

Purification and purity of the peptide were carried out by high performance liquid chromatography (HPLC) and reverse phase HPLC (RP-HPLC). Further identify of molecular weight was performed using electrospray ionization mass (ESI-MS). The sequence of the peptide was identified by Edman method. LFM23 was stored in freeze-dried form at -20°C and dissolved on the day it was used.

Analysis of antibacterial activity of LFM23

Antibacterial activities were tested against one Gram$^+$ bacteria (*S. aureus* 25923) and three Gram$^-$ bacteria (*E. coli* ATCC25922, *Salmonella* ATCC12291 and *P. aeruginosa* ATCC27853). In addition, a fungus strain *P. pastoris* GS115 was also tested. The minimal inhibition concentration (MIC) was determined by broth dilution method with MIC expressed as the lowest final concentration of the peptide, at which more than 50% growth was inhibited. A stock solution of LFM23 was serially diluted at 10 times the required test concentration in 0.01% acetic acid and 0.2% bovine serum albumin (BSA) in polypropylene tubes. The tested strains cultured overnight were recovered by centrifugation at 4200 rpm for 10 min and washed twice with Phosphate-buffered saline (PBS) (0.8 g/L NaCl, 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 1.15 g/L Na_2HPO_4, pH 7.2).

Finally, the cells were diluted in a range of 2×10^5 to 7×10^5 cfu/ml with Mueller-Hinton broth (MHB) medium. Aliquots (100 µL) from each strain suspension were distributed to a 96-well polypropylene microtiter plate, and each well was added 11 µL of LFM23 of various concentrations. Cultures were grown for 18 h with vigorous shaking and cell growth was evaluated by measuring the culture absorbance at 490 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader. All experiments were performed in triplicate.

Determination of hemolysis activity of LFM23

The hemolytic activity of LFM23 was determined using human erythrocyte as described by Maher and McClean (2006). The human erythrocytes were prepared from 4 ml freshly collected human blood by centrifugation at 1500 rpm for 10 min at 4°C. The cells were then washed three times with PBS and resuspended in 4% (v/v) in PBS. The cells were then incubated with selected peptide concentrations for 1 h at 37°C, and finally centrifuged at 3500 rpm for 5 min.

The absorbance of the supernatant was measured at 414 nm with an ELISA plate reader. Zero hemolysis and 100% hemolysis were determined in PBS and Triton X-100, respectively. There were three parallel samples in each concentration and control groups. The hemolysis percent of LFM23 was calculated as follows:

$$\text{Hemolysis (\%)} = \frac{\text{Peptide treatment group } A_{414} - \text{PBS } A_{414}}{\text{Triton } A_{414} - \text{PBS } A_{414}} \times 100\%$$

RESULTS

Design and molecular characterization of LFM23

The designed hybrid peptide LFM23 contained 23 amino

Table 1. The molecular characterization of LFM23.

Parameter	Amino acid sequence	Molecular weight (Da)	Positive charge	pI	Content of α-helix
LFM23	FKCRRWQWRW KKLGARLKVLTTG	2918.5	8	12.02	43%

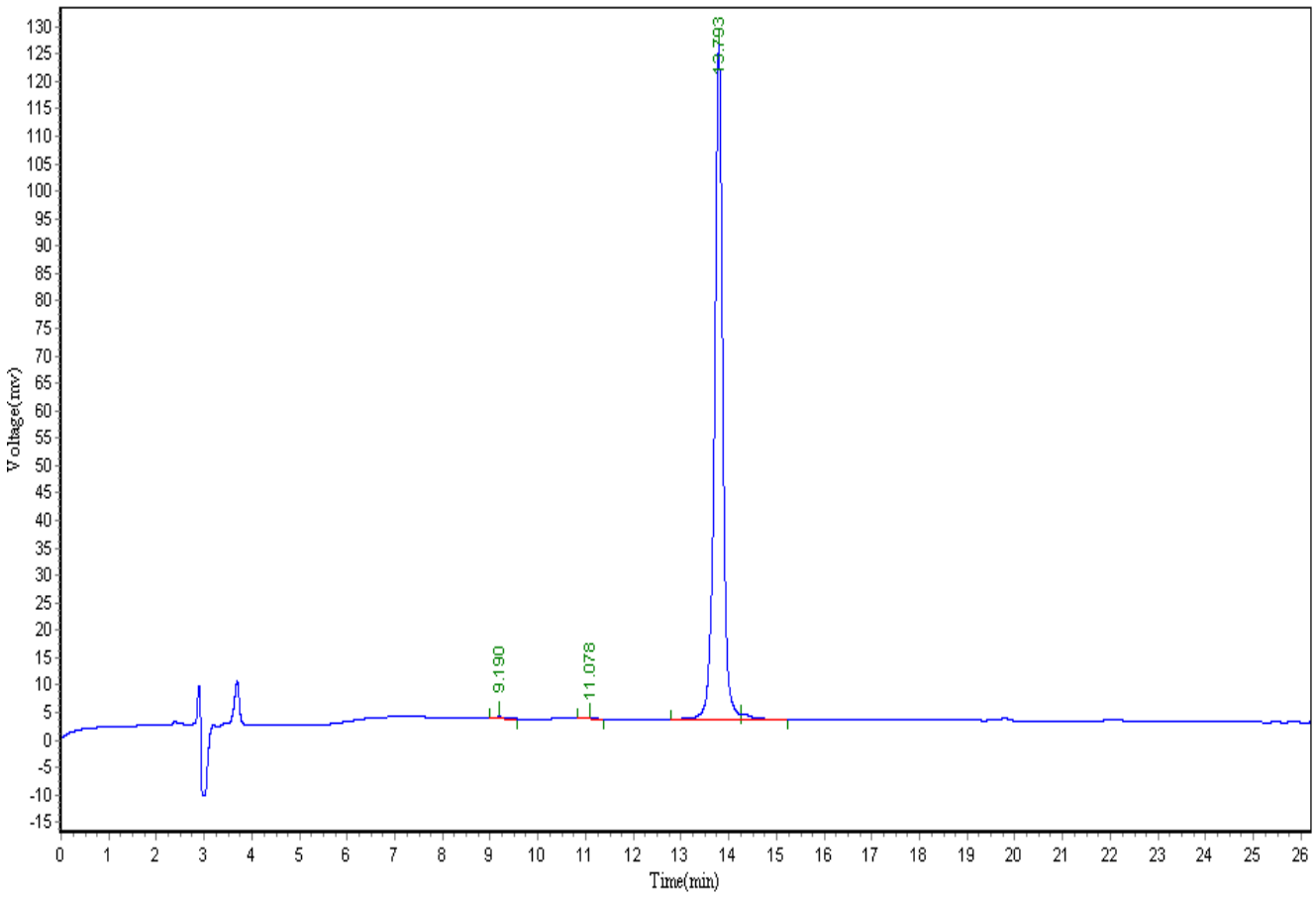

Figure 1. Analysis of LFM23 purity by RP-HPLC. The purity of LFM23 was determined by RP-HPLC on a Venusil ASB-C18 column (150 mm × 4.6 mm, 5 μM), which was pre-equilibrated in 0.1% trifluoroacetic acid (TFA). The mobile phase A (0.1% TFA in acetonitrile) was linearly increased from 22 to 47% in 25 min, while the mobile phase B was 0.1% TFA in water. The detection wavelength was 220 nm.

acid residues (FKCRRWQWRWKKLGARLKVLTTG). The main molecular characterization of LFM23 is listed in Table 1. According to the calculated results of the biologic software ProtParam, the peptide has 8 positive charges with pI 12.02 and its molecular weight was 2918.5 Da. The α-helix structure is 43% of the secondary structure of LFM23. LFM23 is endowed with the main characteristics of AMPs and has the potential to become AMPs. Hence, it could be defined as the cationic AMP.

Synthesis and identify of LFM23

Solid-phase synthesis chemical method was performed to synthesize LFM23. The experimental result showed that the ratio of the coupling synthesis of each amino acid was over 92%, most of them were over 97%. After cleavage of the peptide from resin, the yield of crude LFM23 was about 51.1% and the purity of LFM23 was about 30.2%. The crude product was purified by prepared HPLC and RP-HPLC, and the total yield was 21.9% with the purity of more than 98% (Figure 1). The molecular weight of the product determined by ESI-MS was 2918.57 (Figure 2), which was well corresponded to the theoretical molecular weight of LFM23. The results of amino acid sequencing showed that the N-terminal sequence of LFM23 was FKCRR. Taken together, we concluded that the synthesized LFM23 had the predicted

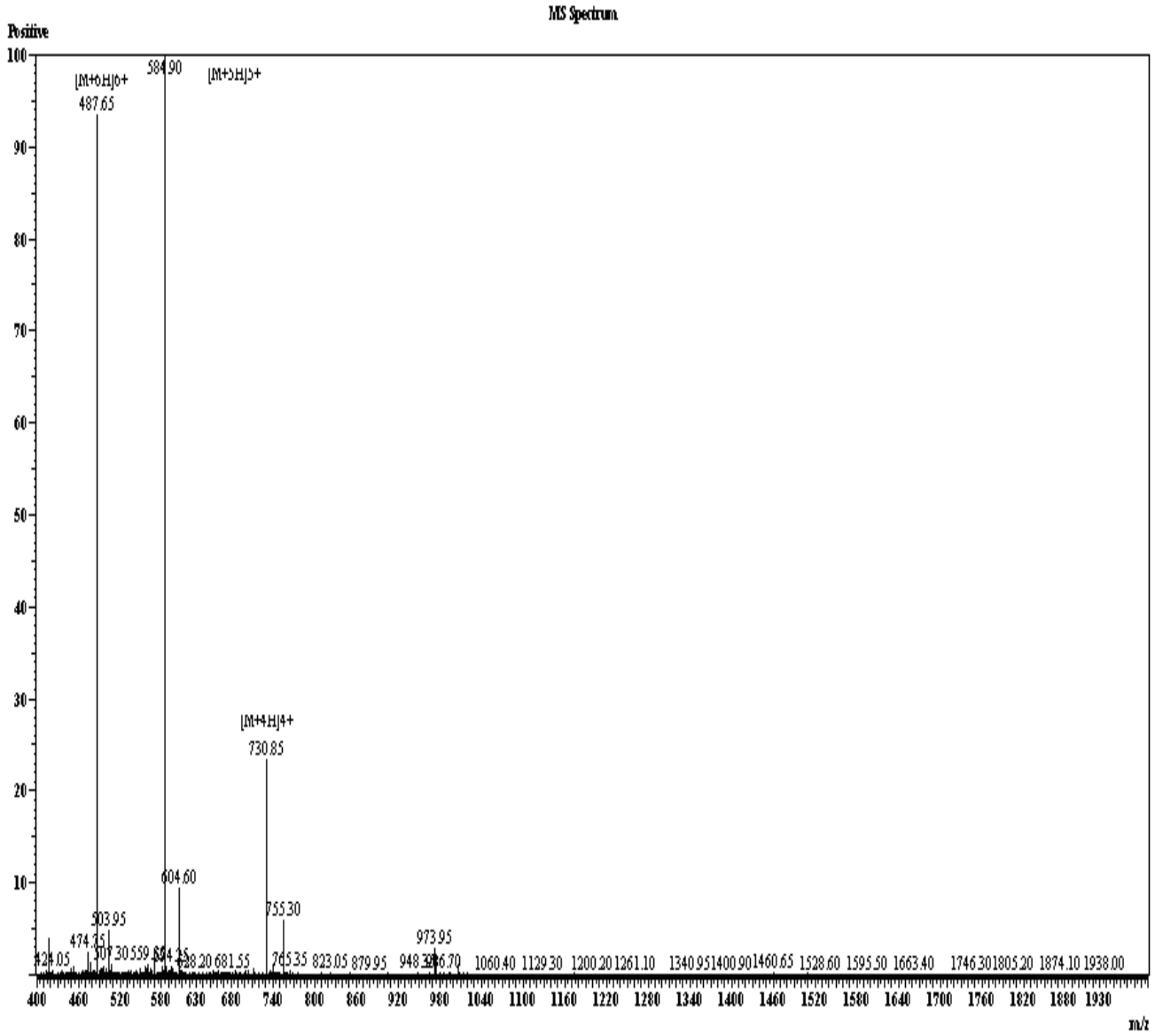

Figure 2. Molecular weight analysis of LFM23 by ESI-MS. The MS working conditions were as follows: ionization mode: ESI; probe bias voltage: +4.5 kV; nebulizer gas (N_2) flow: 1.5 L/min; detector voltage: 1.5 kV; CDL voltage: -20.0 V; CDL temperature: 250°C; block temperature: 200°C.

amino acid sequence.

Antibacterial activity of LFM23

To further determine the activity of LFM23, LFM23 was investigated on inhibition against several strains by broth dilution method. The antibacterial activity of LFM23 against tested strains is reported in Table 2. The results showed that five strains were subject to different degrees of growth inhibition at the different peptide concentration, and the antibacterial effects of LFM23 on different strains were different. According to the definition of MIC, the MIC values of LFM23 on *E. coli* ATCC25922, *S. typhimurium* ATCC12291 and *S. aureus* ATCC25923 were 32 μg/ml, followed by 64 μg/ml of the MIC value on *P. aeruginosa* ATCC27853. The MIC value on *P. pastoris* GS115 was 256 μg/ml. This showed that LFM23 possessed a broad spectrum of antibacterial activity.

Hemolysis of LFM23

The hemolytic activity of LFM23 on human erythrocyte was determined and described in Table 3. LFM23 showed an increasing hemolytic activity as the peptide

Table 2. The MICs of LFM23 on the tested strains.

Strain	MIC (μg/ml)
E. coli ATCC25922	32
S. typhimurium ATCC12291	32
P. aeruginosa ATCC27853	64
S. aureus ATCC25923	32
P. pastoris GS115	256

Table 3. Hemolysis rate of human erythrocyte at different concentrations of LFM23.

Concentration of LFMA23 (μg·ml^{-1})	Hemolysis rate (%)
4	0.131 ± 0.0044
8	0.243 ± 0.0033
16	0.332 ± 0.0048
32	0.813 ± 0.012
64	1.223 ± 0.0233
128	1.467 ± 0.0089
256	1.727 ± 0.012
512	2.077 ± 0.0088

concentration increased, but the overall hemolytic activity was very weak, and hemolysis rate was 2.077% even at 512 μg/ml of LFM23. Therefore, it can be suggested that LFM23 did not produce hemolysis on human erythrocyte.

DISCUSSION

In nature, almost all kinds of plants, animals and microorganisms produce AMPs which are natural biological barriers against harm from invasion pathogens. The continuous use of antibiotics has resulted in the emergence of resistant bacteria strains. In recent years, AMPs have received much attention as pharmaceutical applications. Several hundred AMPs have been isolated and identified. However, most of AMPs cannot meet pharmaceutical demands due to some activity deficiency of AMPs such as narrow antimicrobial spectrum, low activity, hemolysis, etc (Marr et al., 2006).

Nevertheless, to improve the activity of AMPs, molecular design to obtain novel AMPs is usually conducted based on the primary structures of natural AMPs. Numerous studies have been carried out on the structure–activity relation of LfcinB and its derivatives. Rekdal et al. (1999) and Strøm et al. (2000) synthesized a derivative LfcinB15 consisting of residues 1 to 15 of LfcinB. It has only minor loss of antimicrobial activity relative to the native peptide LfcinB. Tryptophan (Trp) is thought to function as an anchor in membrane proteins (Schiffer et al., 1992). Trp is also suggested to act as a needle that pulls AMPs across phospholipid membranes (Wimley and White, 2000). Most of the resulting peptides by the incorporation of one ore more additional Trp displayed a substantial increase in the antimicrobial activity. LfcinB15 generated by replacing the residue Met 10 with Trp showed improved activity (Rekdal et al., 1999; Strøm et al., 2002). In the present study, a novel AMP LFM23 was designed according to LfcinB and melittin. According to the results predicted by the biological software, LFM23 integrated with the truncated sequences of LfcinB and melittin has the molecular characterization of cationic AMPs. LFM23 obtained by solid-phase synthesis had high purity above 98%, which can be used to determine the activity and molecular structure of LFM23.

In order to measure the biological activity of LFM23, antibacterial experiments were carried out. The results showed that LFM23 had apparent antibacterial activity against G^+ and G^- bacteria. It also had some resistance against yeast, although the activity was not high. Interestingly, LFM23 did not show hemolytic activity to the erythrocyte. It is well know that melittin has strong hemolytic activity, which brought many problems to the use of melittin. However, LFM23 retained the antibacterial activity of LfcinB and melittin while eliminating the hemolysis of melittin. The results therefore indicated that it could be an effective way to hybrid several AMPs to obtain novel AMPs molecules with improved antibacterial activity.

In conclusion, the truncated sequences of LfcinB and melittin were designed to integrate and generate a novel AMP, LFM23. LFM23 was successfully obtained by solid-phase synthesis techniques with more than 98% purity. Moreover, LFM23 showed significant inhibitory effects against *E. coli*, *S. typhimurium*, *S. aureus*, *P. aeruginosa* and yeast without producing hemolysis. Therefore, the peptide could be used as alternative to medical agents. However, further investigations need to be carried out on molecular structure, action mechanism and immunogenicity of LFM23.

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