MJ347-81F4 A & B, Novel Antibiotics from *Amycolatopsis* sp.: Taxonomic Characteristics, Fermentation, and Antimicrobial Activity

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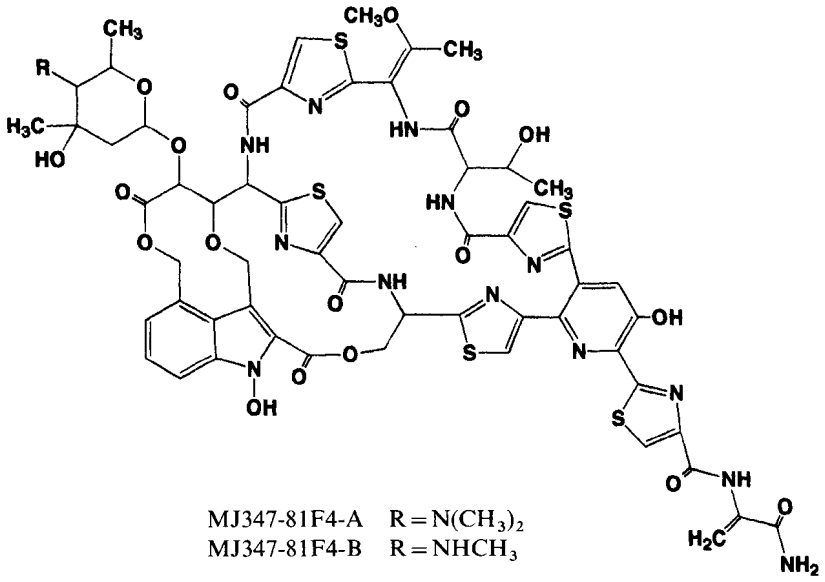
Strain MJ347-81F4 has been found to produce two new cyclic thiazolyl peptide antibiotics, components A and B. Taxonomic studies including morphological and physiological characteristics and chemical analysis of whole cells of the producing strain revealed this microorganism to belong to genus *Amycolatopsis*, and so we designated the strain *Amycolatopsis* sp. MJ347-81F4. After 10 to 12 days of fermentation, most of the antibacterial activity was present mainly in the mycelial cake and reached its maximum level. In comparison with reference compounds, A as the major component showed excellent *in vitro* activity against Gram-positive bacteria including highly methicillin-resistant *Staphylococcus aureus* (MRSA) and *Enterococcus faecalis* with MICs in the range of concentration of $0.006 \sim 0.1$ μg/ml. The results on the antimicrobial activity against thiazolyl peptide-resistant mutants of *Bacillus subtilis* NRRL B-558 indicated that the possible molecular target of MJ347-81F4 component A might be the 50S subunits of the ribosome, the inactivation of which would inhibit protein synthesis.

During the course of our investigation for the discovery of novel antibacterial antibiotics, especially those possessing potent activity against methicillin-resistant *Staphylococcus aureus* (MRSA), from microorganisms isolated from new soil samples, we found *Amycolatopsis* sp. MJ347-81F4 to produce two new antibiotics, hereafter designated MJ347-81F4 components A and B. The antibiotic complex was mainly present in the mycelial cake and it was extracted with acetone. The respective components were purified by solvent extraction and bioassay-directed fractionation employing a combination of repetitive countercurrent chromatography and preparative reversed-phase HPLC. The structures of components A and B with molecular formulas of $C_{61}H_{60}N_{14}O_{18}S_5$ (MW: 1437) and $C_{60}H_{58}N_{14}O_{18}S_5$ (MW: 1423) were elucidated by chemical degradation and spectroscopic methods including a combination of extensive 2D-NMR and MS experiments. As shown in Fig. 1, components A and B are new members of the family of thiazole-containing cyclic peptides, as described in BERDY[1]. These structures resemble closely that of glycothiohexide in sharing a thiazolyl peptide core of similar size and a dimethylamino sugar. The major difference between MJ347-81F4 components A and B and glycothiohexide is the presence of dehydroalanine in the former two and of cysteine derived moiety as the bicyclic bridgehead in the place of the serine derived moiety in the latter. The major component A was isolated in sufficient quantity to permit evaluation of its biological activity. Accordingly, in this paper, we describe the

Fig. 1. Structure of MJ347-81F4 components A and B.



MJ347-81F4-A R = $N(CH_3)_2$
MJ347-81F4-B R = $NHCH_3$

taxonomic studies of the producing strain, fermentation, and biological activities of MJ347-81F4 component A. The isolation procedure, physico-chemical properties, and structural elucidation will be described in a separate paper.

Materials and Methods

Microorganisms

Strain MJ347-81F4 was isolated from a soil sample collected in Hamochi-machi, Sado-gun, Niigata prefecture, Japan. The strain has been deposited in the National Institute of Bioscience and Human-Technology, Japan, under the accession number FERM BP-5184. *Amycolatopsis orientalis* ISP 5040^T and *Nocardia asteroides* IFO 3384^T were used as reference strains for detection of mycolic acid. Two thiazolyl peptide-resistant mutants of *Bacillus subtilis* NRRL B-558 were selected by one overnight incubation of a shaking-culture[13]. The MRSA strains used in this study were clinical isolates obtained in Japan in 1992. All of the other strains used belong to the culture collection of our laboratory at Taiho Pharmaceutical Co., Ltd.

Taxonomic Studies

The media and procedures used for cultural and physiologial characteristics of this strain were those described by SHIRLING and GOTTLIEB[2], and by WAKSMAN[3]. The culture was carried out at 27°C for 2 to 4 weeks. The color index was assigned in accordance with color chips from the Color Harmony Manual (Container Corporation of America). The temperature range for growth was determined on inorganic salts-starch agar (ISP No. 4 medium). Utilization of carbon sources was examined by the method of PRIDHAM and GOTTLIEB[4]. The specimens for observation of spore morphology were prepared by use of a modified method of LOCCI[5] and photographed by a scanning electron microscope (Model Hitachi S-570).

Chemotaxonomic Studies

Strain MJ347-81F4 was cultured in 100 ml of YD medium consisting of 1.0% yeast extract and 1.0% glucose in 500-ml Erlenmeyer flasks at 30°C on a rotary shaker (220 rpm). After incubation for 7 days, the mycelial cake was harvested by centrifugation, and lyophilized to dryness after having been washed with water. The type of diaminopimelic acid and sugars in the whole-cell hydrolysates was determined by the method of BECKER *et al.*[6] and LECHEVALIER and LECHEVALIER[7], as modified by STANECK and ROBERTS[8], for separation on a cellulose thin-layer chromatograph. Phospholipids and mycolic acids were extracted and then analyzed by the procedure of MINNKIN *et al.*[9,10]. Menaquinones, after extraction and purification according to the method of COLLINS *et al.*[11], were analyzed by HPLC on an Inertsil ODS-2 column (150 × 4.6 mm, i.d., GL Sciences) under the conditions described by TAMAOKA *et al.*[12], and further followed by electron impact mass-spectrometry. Fatty acid-pattern was analyzed by gas liquid chroma-

tography of whole-cell methanolysates. Their methyl-esters were determined by use of a Shimadzu model GC-17A gas chromatograph equipped with a flame ionization detector, and a Shimadzu model CR-6A integrator. A Megabore DB-1 column (15 m × 0.35 mm, i.d., J & W Scientific) was used.

Fermentation

Seed medium consisting of 1.0% glycerol, 2.0% galactose, 2.0% dextrin, 1.0% Bacto soytone, 0.5% corn steep liquor, 0.2% $(NH_4)_2SO_4$, and 0.2% $CaCO_3$ was adjusted to pH 7 and then sterilized at 121°C for 15 minutes in 500-ml Erlenmeyer flasks containing 100 ml of medium. The culture on an agar slant was inoculated into each flask and cultured at 27°C for 4 days on a rotary shaker. Stock for the seed culture was prepared as follows: one ml of 20% glycerol was added to 1 ml of the seed culture and stored at −35°C. Then, stock culture was inoculated to the seed medium and cultured at 27°C for 3 days under the same conditions. Five milliliters of the seed culture thus obtained was inoculated into each of several 500-ml Erlenmeyer flasks, each containing 120 ml of the same medium. The fermentation was carried out at 27°C for 11 days. Mycelial growth was expressed as packed cell volume obtained from 10 ml of the culture fluid after centrifugation at 2,800 rpm for 10 minutes.

In Vitro Susceptibility Testing and HPLC Analysis

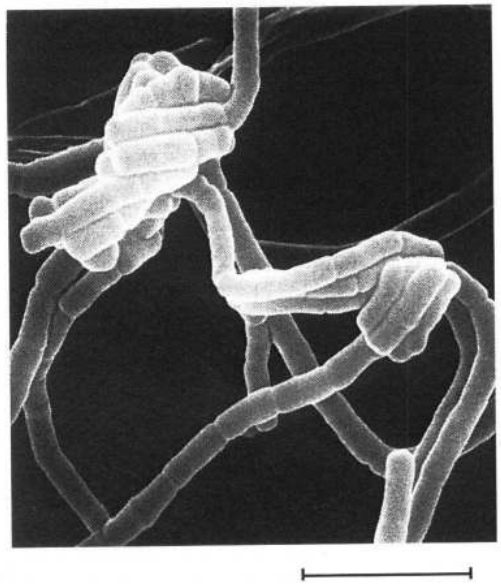
Antimicrobial activity was monitored as growth inhibition against *Staphylococcus aureus* FDA 209P by the paper disk method. Further, the potency of major component A in the fermentation broth was determined by reversed-phase HPLC using an Inertsil ODS-2 column with acetonitrile/ tetrahydrofuran/0.01% trifluoroacetic acid (22 : 10 : 68) at a flow rate of 1.0 ml/minute with detection by UV absorption at 220 nm. The sample for assay by HPLC was prepared as follows: 2 ml of acetone was added to 1 ml of the fermentation broth; and after having been stirred vigorously for 1 minute, the mixture was centrifuged at 3,100 rpm for 5 minutes. Ten microliters of the supernatant obtained was directly applied to a Shimadzu Model LC-6A HPLC system. One unit of antibiotic production was defined as the amount of component A that gave peak area of 1,000 under the HPLC conditions as mentioned above.

Antimicrobial Activity

Antimicrobial spectrum was determined by the serial agar dilution method on Mueller-Hinton agar (Difco). Approximately 10^4 CFU per spot were inoculated onto agar plates that contained two-fold serial dilutions of antibiotic. Minimum inhibitory concentration (MIC) was indicated as the lowest concentration of antibiotic that inhibited completely visible growth after incubation for 18 hours at 37°C. Further, looking for possible molecular targets, we measured the MIC of MJ347-81F4 component A in comparison with that of the reference compounds against *Bacillus subtilis* NRRL B-558 ATM^r (V228A) and TS^r (L11) mutants, which are resistant to amythiamicin (MIC, >100 μg/ml) and thiostrepton (MIC, >100 μg/ml), respectively, as described previously[13].

Fig. 2. Scanning electron micrograph of spores of strain MJ347-81F4 on diluted yeast extract-starch agar.

The bar represents 2.5 μm.



Results and Discussion

A scanning electron micrograph of spores of strain MJ347-81F4 is shown in Fig. 2. This strain displayed branched vegetative hyphae, which had a slight tendency to assume a zig-zag shape and tended to break down into squarish subunits. The aerial hyphae produced cylindrical conidia with straight to flexuous chains. The spores were 0.4 ~ 0.5 × 0.9 ~ 1.2 μm in size with a smooth surface. Sporangia, motile spores, flagellum, and synnemata were not observed.

The culture characteristics of strain MJ347-81F4 are summarized in Table 1. The substrate mycelium showed a yellow to light yellowish-brown color on various media.

Table 1. Culture characteristics of strain MJ347-81F4.

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	Yellowish-brown (2 ne)	Yellowish-white	Absent
Glucose-asparagine agar	Pale yellow	Yellowish-white	Absent
Glycerol-asparagine agar (ISP med. No. 5)	Pale yellow	Yellowish-white	Absent
Inorganic salts-starch agar (ISP med. No. 4)	Light yellowish-brown (2 pg, 3 pg)	Pale yellow (1 ba) ~yellowish-white (1 ca)	Absent
Tyrosine agar (ISP med. No. 7)	Pale yellow~light yellowish-brown (2 le)	Yellowish-white	Yellowish
Oatmeal agar (ISP med. No. 3)	Dull yellow (1 1/2 ic)	Yellowish-white	Yellowish
Yeast extract-malt extract agar (ISP med. No. 2)	Light yellowish-brown (2 le)	Yellowish-white	Absent
Nutrient agar	Light yellowish-brown	Whitish	Absent
Bennet's agar	Light yellowish-brown (2 le)	Whitish	Absent

Table 2. Physiological characteristics of strain MJ347-81F4.

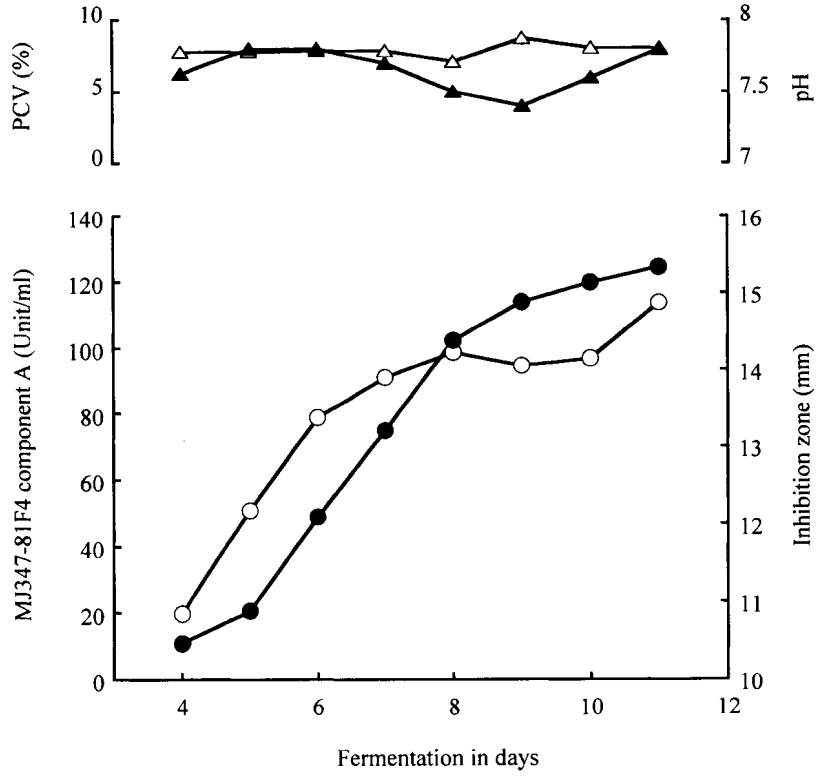
Temperature range for growh		27~37°C
Optimum temperature		27°C
Formation of melanoid pigment		Negative
Liquefaction of gelatin		Positive
Coagulation of milk		Negative
Peptonization of milk		Positive
Hydrolysis of starch		Positive
Reduction of nitrate		Negative
Decomposition of cellulose		Negative
Production of H_2S		Negative
Growth in NaCl		1~5 (%)
Utilization of carbon sources	Positive	D-Xylose, D-glucose, D-fructose, sucrose, D-galactose, maltose, soluble starch, glycerol
	Negative	L-Arabinose, L-rhamnose, inositol, raffinose, D-mannitol, salicin

Aerial mycelia assuming yellowish-white color were observed on some of the synthetic media. Melanoid pigments and other soluble pigments were not found. The physiological characteristics and utilization of carbon sources of this strain are shown in Table 2. This strain utilized D-xylose, D-glucose, D-fructose, sucrose, D-galactose for growth, but not L-arabinose, L-rhamnose, raffinose, inositol, D-mannitol, or salicin.

The results of chemotaxonomic studies were as follows: Analysis of the whole-cell hydrolysate demonstrated the presence of *meso*-diaminopimelic acid, arabinose, and galactose. Accordingly, this strain was classified as having type-IV cell walls and a type-A whole-cell sugar pattern, according to the classification proposed by LECHEVALIER and LECHEVALIER[7]. Further, analysis of the whole-cell phospholipids revealed the presence of diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), belonging to type-PII. Mycolic acids were not detected. The predominant isoprenoid quinones were MK-9(H_4), and a slight amount of MK-9(H_2) was also detected. This strain contained major amount of *iso*-branched 13-methyltetradecanoic acid (*iso*-15:0), *cis*-9,10-dehydrohexadecanoic acid (*cis*-16:1), *cis*-9,10-dehydroheptadecanoic acid (*cis*-17:1) as well as other minor components. The taxonomic properties mentioned above and particularly the chemotaxonomic results were in good accordance with those of genus *Amycolatopsis*[14~15], and so we designated this micro-

Fig. 3. Typical time course of MJ347-81F4 component A production.

● MJ347-81F4 component A (Unit/ml), ○ inhibition zone (mm) with paper disk method, ▲ pH, △ packed cell volume (%).



organism as *Amycolatopsis* sp. MJ347-81F4, although the species was not determined.

A typical time course for the production of component A is shown in Fig. 3. As seen, the maximum yield (125 units/ml) was obtained at culture day 11. The presence of component A in the culture broth was much less than a quarter of that in the mycelia. The antibacterial activity, as estimated by the inhibitory zone against *S. aureus*, paralleled the production of component A. Next we tested the productivity in the A-1 and F-1 medium used in glycothiohexide production[22], because component A resembled closely glycothiohexide in structure as mentioned above. As a result, the maximum production of component A in the A-1 and F-1 medium was less than 1/4 and 1/2, respectively, of the amount in the medium used for component A.

Antibiotics MJ347-81F4 components A and B are structurally related to cyclic thiazolyl peptides and have some structural similarity to these antibiotics. Compounds with closely related structures such as A-10255[16], glycothiohexide[17], S-54832[18], GE-2270[19], thiostreptons[20], and amythiamicin[21] have been found, and are produced by *Streptomyces* sp., *Sebekia* sp., *Micromonospora* sp., *Planobispora rosea*, *Streptomyces azureus*, and *Amycolatopsis* sp., respectively. There are also examples of other similar compounds produced by microorganisms of different taxa. Accordingly, it seems that the biosynthesis system of thiazolyl peptide antibiotics occurs widely in microorganisms.

Antibiotic MJ347-81F4 component A showed excellent *in vitro* activity against Gram-positive bacteria including methicillin-resistant *S. aureus* (MRSA) and *E. faecalis*, but was inactive against most Gram-negative bacteria (Table 3). These results are similar to those reported for other thiazolyl peptide antibiotics[16~21]. Further, the *in vitro* antibacterial activity against various clinical isolates of MRSA, which exhibited an MIC of $>50\,\mu g/ml$ toward methicillin, was compared with that of the reference compounds such as vancomycin. As shown in Fig. 4, component A was the most active of the compounds, even in comparison with the reference compound, vancomycin, which is well known to be one of the most useful antibiotics for chemotherapy of MRSA infections. However, the slow bactericidal activity and side effects of vancomycin hinder effective treatment of MRSA infections[23]. These results indicated that

MJ347-81F4 component A as well as thiazolyl peptides may be promising candidates as anti-MRSA drugs. Furthermore, it has been recently reported that plasmid-mediated *E. faecalis* and *E. faecium* resistant to vancomycin pose serious clinical problems[24,25]. It will be interesting to determine whether these components will affect these strains or not. The evaluation of the *in vivo* efficacy of MJ347-81F4 component A against MRSA and *E. faecalis* is now in progress. The antifungal spectrum of MJ347-81F4 component A was determined by the broth microdilution method using RPMI 1640 medium after a 40-hour incubation at 37°C. It was active with MICs value of 50, 50, 25, 25 μg/ml, respectively, against *Candida krusei*, *C. pseudotropicalis*, *C. glabrata* and *Saccharomyces cerevisiae*, but inactive against filamentous fungi.

Next we evaluated the mechanism of action of component A by assessing the effect of the compounds on thiostrepton (TS)- and amythiamicin (ATM)-resistant *B. subtilis* NRRL B-558. The thiazolyl-peptide antibiotics such as thiostrepton, thiopeptin, and siomycin are known to act on 50S ribosomal subunits, consequently inhibiting

Table 3. Antimicrobial spectrum of MJ347-81F4 component A.

Microorganisms	MIC (μg/ml)
Staphylococcus aureus FDA 209P[a]	0.1
S. aureus Smith[a]	0.1
S. aureus 70[b]	0.05
S. aureus 92-1045[b]	0.1
S. aureus 92-1191[b]	0.1
S. epidermidis IFO 3762	0.2
Micrococcus luteus ATCC 9341	0.006
Enterococcus faecalis ATCC 21212	0.1
Escherichia coli NIHJ JC-2	0.1
Citrobacter freundii ATCC 8090	>50
Klebsiella pneumoniae NCTC 9632	>50
Proteus mirabilis IFO 3849	>50
Serratia marcescens IFO 12648	>50
Pseudomonas aeruginosa 46001	>50

Minimum inhibitory concentration (MIC) of methicillin against methicillin-resistant *S. aureus* (MRAS) was 50 μg/ml.
[a] Standard strain, [b] clinical isolate-MRSA.

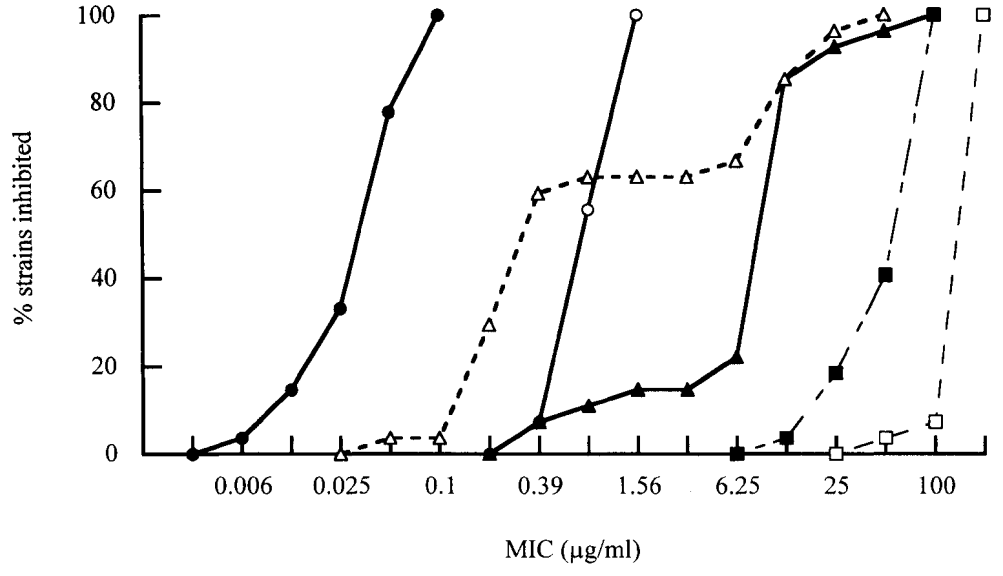
Table 4. Antimicrobial activity of MJ347-81F4 component A against thiazolyl peptide antibiotic-resistant mutants.

Drug	MIC (μg/ml)		
	Wild type	TS^r	ATM^r
MJ347-81F4 A	<0.19	>100	<0.19
Thiostrepton	<0.19	>100	<0.19
Siomycin	<0.19	>100	<0.19
Amythiamicin	<0.19	<0.19	>100

Wild type: *Bacillus subtilis* B-558.
TS^r: Thiostrepton-resistant mutant (L11) of *B. subtilis* B-558.
ATM^r: Amythiamicin-resistant mutant (V228A) of *B. subtilis* B-558.

Fig. 4. Antimicrobial activity of MJ347-81F4 component A and reference compounds against clinical isolates of methicillin-resistant *Staphylococcus aureus*. (27 strains).

● MJ347-81F4 component A, ○ vancomycin, ▲ ofloxacin, △ minocycline, ■ flomoxef, □ methicillin.



protein synthesis. Very recently, HORI *et al.*[13] reported that amythiamicin inhibited polyU-directed poly-(phe) synthesis in a cell-free synthesis system including 50S ribosomal subunits. As shown in Table 4, component A as well as thiostrepton and siomycin showed poor antibacterial activity against *B. subtilis* TS^r L-11 (MIC, $>100\,\mu g/ml$), but was active against *B. subtilis* ATM^r V228A (MIC, $<0.19\,\mu g/ml$). The possible molecular target of this antibiotic may be the 50S ribosomal subunits to inhibit protein synthesis, since component A showed cross-resistance with thiostrepton.

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