

## CHAPTER 31

**Antibiotic Production by *Streptomyces cinnamonensis* ATCC 12308**

The ability of *S. cinnamonensis* ATCC 12308 to produce polyether type antibiotics was investigated. This organism was found to produce both whole-broth antibiotic activity and methanol-extractable antibiotics. The addition of  $\text{Fe}_2\text{SO}_4$  increased antibiotic production. Methyl propionate and ethyl acetate enhanced antibiotic production. The methanol extracts were active against *Staphylococcus aureus*, *Micrococcus luteus*, *Streptococcus faecalis*, *Bacillus megaterium*, *Alcaligenes faecalis*, *Pseudomonas aeruginosa*, and *Pseudomonas maltophilia*. Thin-layer chromatographic results indicated that the active component in the methanol extracts differs from several authentic samples of polyether antibiotics.

## INTRODUCTION

Polyether antibiotics have proven to be of great value for the prevention of coccidiosis in poultry and other animals (Reid et al. 1977; Reid et al. 1975) and the increase of feed-efficiency ratios in poultry and beef cattle. These antibiotics are monocarboxylic acid ionophores with furan-pyran polyether structures. In the crystalline state the two ends of the antibiotic molecule are held together by a hydrogen bond between the carboxyl group and a tertiary hydroxyl on the terminal tetrahydropyran ring to form a cyclic structure. Because all of the oxygen functions are concentrated in the center of the molecule and the hydrophobic alkyl groups are on the surface, the polyether antibiotics are almost insoluble in water and soluble in organic solvents in either the salt or free acid forms (Westley 1977). Biologically, the polyether antibiotics have broad spectrum activity against gram-positive bacteria, yeasts, fungi, and protozoa but are too toxic for most therapeutic uses. The inclusion of small amounts of some of the polyether antibiotics, such as monesin in the forage fed to steers, results in an increase in feed efficiency. It was discovered by Dinius et al. (1976) that this increase resulted from an increase in the ratio of propionic acid to acetic acid in the ruminal content.

At least nine species of *Streptomyces* produce polyether antibiotics and appear to comprise a class of fairly closely related organisms. *Streptomyces cinnamonensis* (ATCC 15413), which produces monensins A, B, C, and D (Haney and Hoehn 1968), was isolated from soil and classified as a strain of *Streptomyces cin-*

*namonensis* Okami, NRRL B1588 by C. E. Higgons (Stark et al. 1968). The spore chain morphology of ATCC 15413 was described (Haney et al. 1964) as having straight to flexuous sporophores and spore chains with neither spirals nor hooks present, which differs from the description by Pridham and Tresner (1974), who described *S. cinnamoneus* spore chains as forming hooks, open loops, or greatly expanded coils. The literature is further confused by the existence of several species with very similar names and descriptions, such as *Streptomyces cinnamoneus*, which is now classed as *Streptoverticillium cinnamoneum* (Baldacci and Locci 1974) and *Streptoverticillium cinnamoneum* forma *azacoluta*. The ambiguities concerning the possible coidentity of *S. cinnamoneus* ATCC 15413 and *S. cinnamoneus* ATCC 12308 was the subject of this study. Classic taxonomical approaches were planned for one study and in the other the ability of the two strains to produce polyether antibiotics would be compared. This paper reports the results of study of antibiotic production by *S. cinnamoneus* ATCC 12308.

#### MATERIALS AND METHODS

**Cultures.** *Streptomyces cinnamoneus* ATCC 12308 and *Streptomyces cinnamoneus* ATCC 15413 were obtained from the American Type Culture Collection, Rockville, MD. Slant cultures were grown for 5 d on the agar medium of Stark et al. (1968) consisting of glucose, 10 g; Soytone (Difco), 10 g; agar, 25 g; and distilled water 1,000 ml. The pH was adjusted to 7.3 with 1 N NaOH before sterilization by autoclaving. Spores were harvested from the slants to make suspensions for inoculation of the culture media.

**Culture media.** The media used in this study were based on those of Stark et al. (1968) and are described in Table 1.

TABLE 1. *Basal media used during fermentation studies*

Medium component	Concentration (g/liter)		
	1	2	3
Glucose	5.0	30.0	10.0
Nutrisoy flour	15.0		
Soytone (Difco)			15.0
Soy grits		25.0	
Refined soybean oil		20.0	
Methyl oleate		20.0	
Dextrin	20.0		20.0
Yeast extract (Difco)	2.5		2.5
CaCO <sub>3</sub>	1.0	1.0	1.0
KCl	0.10	0.1	0.1
K <sub>2</sub> HPO <sub>4</sub>	0.10	0.1	0.1
MnCl <sub>2</sub> ·4H <sub>2</sub> O		0.6	0.6
Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub>		0.3	0.3
pH	8.0	8.0	7.0

**Vegetative inoculum.** Vegetative inocula were grown as described by Stark et al. (1968).

*Growth.* Mycelial growth was measured as packed cell volume by centrifuging a 10-ml sample of whole-broth culture for 30 min at  $500 \times g$ . Growth was recorded as percentage of solids (v/v).

*Comparison of antibiotic production by S. cinnamonensis ATCC 15413 and S. cinnamonensis ATCC 12308.* Cultures of *S. cinnamonensis* ATCC 15413 and *S. cinnamonensis* ATCC 12308 were grown in the Stark et al. (1968) production medium (Medium 2, Table 1) using a 2% inoculum from the vegetative culture. The cultures were incubated at 32 C for periods of several days. Values for pH, packed cell volume, viable cell number, whole-broth antibiotic activity, and methanol extractable antibiotic activity were measured on at least a daily basis. The methanol extractable activities reached maxima after approximately 4 d incubation. This incubation period was, therefore, selected as the standard for comparison and the results that are reported and discussed in the results section are those obtained after 4 d incubation.

*Assay.* Antibiotic titers in fermentation broth samples were measured by microbiological assay on Tryptic Soy Agar (TSA, Difco). Antibiotic titers were calculated as micrograms per milliliter assayed against monensin standards. Five replicate assays were made of each sample using 12.5-mm diameter Whatman No. 1 filter paper discs. *Bacillus megaterium* ATCC endospore suspensions ( $2 \times 10^9$  per ml) were used as lawns on TSA, by the Kirby-Bauer technique, 40 ml per  $15 \times 150$ -mm Petri dish. Each disc received exactly 0.10 ml of the mixture to be assayed. After they had dried, five discs for each assay were placed on the inoculated assay plate. The diameters of the inhibition zones were measured with calipers after incubation of the plates for 16 h at 30 C.

Assay samples were prepared as described by Stark et al. (1968). Methanol extracts were prepared by adding 10 ml of methanol to 2 ml of fermentation medium containing both cell mass and product, referred to as whole-broth in the remainder of the paper. Stark et al. (1968) established that this technique reproducibly extracted 90% of monensin activity. After extraction for approximately 30 min, the cells were removed by centrifugation. Assay samples were diluted, when necessary, with 25% (v/v) methanol in distilled water.

*Identification of methanol extractable components.* Authentic samples of monensin Na, A204 A, and dianemycin were provided by Lilly Laboratories. Authentic samples of lasalocid Na, X-205 Na, and nigericin were obtained from Hoffman-La Roche Inc. In addition, a sample of rumensin, which is the commercial product intended as a ruminant feed additive and contains monensins A and B, was obtained from a commercial source. The antibiotic(s) were extracted and crystallized from rumensin as described by Haney and Hoehn (1968).

The methanol extracts were analyzed by thin-layer chromatography on Eastman Chromogram silica gel, without fluorescent indicator, thin-layer chromatography sheets  $20 \times 20$  cm. Samples were applied 2 cm from the bottom of the plates and developed with ethyl acetate. Whatman 3MM,  $20 \times 20$ -cm filter paper sheets were also used as chromatographic supports and were developed with ethyl acetate as described above. Following chromatographic development the dry plates were sprayed with 3% vanillin in 1.5% ethanolic sulfuric acid

(Haney and Hoehn 1968) or 3% H<sub>2</sub>SO<sub>4</sub> and heated in an oven for 5 min at 100 C. Several other reagents including ninhydrin, *p*-dimethylaminobenzaldehyde/antimony trichloride, iodine/sodium azide, nitroprusside, isatin, sulfanilic acid, dinitrosalicylic acid, alkaline silver oxide, aniline/xylose, acridine, and dinitrophenylhydrazine were used to develop the chromatograms and identify the chemical properties of any of the solutes contained in the methanol extracts. There were no ninhydrin or isatin reacting compounds, presumably amino acids or proteins, in the extracts. Dinitrosalicylic acid and alkaline silver oxide, which are tests for sugars, dinitrophenylhydrazine as a test for keto acids, and aniline/xylose and acridine as tests for organic acids all failed to react. The exception was the Ehrlich *p*-dimethylaminobenzaldehyde/HCl reagent (Smith 1969), which was more sensitive than the vanillin reagent in reacting with known polyether antibiotics.

*Antibiotic stabilities.* Aliquots of 50 ml each from ATCC 12308 fermentation broth containing antibiotic activity received the following treatments: none, 50 C for 30 min, 75 C for 30 min, 100 C for 30 min, adjust to pH 2.0 with HCl, adjust to pH 10 with NaOH, and adjust to 0.9 M with trichloroacetic acid. Each aliquot was then assayed for whole-broth and methanol extractable activity.

*Solubility of methanol extractable antibiotics.* Aliquots of 100 ml each of ATCC 12308 fermentation broth were adjusted to pH 9.0, mixed with 3 g of Avicel, and filtered. Each filter cake was extracted four times with 25 ml of one of the solvents.

*Isolation procedure.* Fermentation broth (500 ml) containing active antibiotic activity produced by ATCC 12308 was adjusted to pH 9.0 with NaOH, mixed with 15 g of Avicel, and filtered. The filter cake was repeatedly extracted with CHCl<sub>3</sub> (totalling 200 ml). The residual filter cake following extraction with CHCl<sub>3</sub> was extracted with 150 ml of CH<sub>3</sub>OH. The original 500 ml of filtrate was adjusted to pH 9.0 and extracted with 200 ml of CHCl<sub>3</sub> in a continuous extractor for 24 h. This extract was concentrated *in vacuo* to 25 ml. Each of the three extracts had biological activity.

*In vitro antimicrobial spectrum.* The methanol extract of an active ATCC 12308 culture was placed on either 0.1 or 0.2 ml amounts on 13-mm filter paper discs. The Kirby-Bauer technique was used to inoculate TSA in a 150 × 15-mm Petri dish with each culture. Five antibiotic discs and one 3 μg control of monensin Na were placed on each plate and incubated 24 h at 30 C.

*Effect of medium composition on antibiotic production.* The effect of medium component on the production of whole-broth and methanol extractable antibiotics was investigated by omitting one component at a time from medium 3 in Table 1. The effect of adding 10.0 g of one of the following to medium 3 was determined in a separate study: refined soybean oil (Leveland Vegetable Oil Co.), ethyl acetate (J. T. Baker Co.), methyl deconate (Eastman), methyl formate (Eastman), methyl hexanoate (Eastman), methyl laurate (Eastman), methyl myristate (Eastman), methyl oleate (Eastman), methyl octanoate (Eastman),

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methyl palmitate (Eastman), methyl propionate (Eastman), and methyl stearate (Eastman).

A volume of 100 ml of medium was used per 500-ml baffled Erlenmeyer flask. Each medium was adjusted to pH 7.0 and two flasks per medium were used in the study. The inoculum was 2.0 ml from a 3 d culture of ATCC 12308 in medium 1. The cultures were incubated at 32 C with agitation at 300 rpm (2.5 cm radius). Several preliminary studies indicated that methanol extractable activity peaked in medium 3 after 4 d incubation, and, subsequently, all cultures were harvested at 96 h for this study.

#### RESULTS

The principal questions to be answered in this study were (1) does *S. cinnamomensis* ATCC 12308 produce both whole-broth and methanol soluble antibiotics, and (2) if antibiotics are produced, are they the same as those produced by *S. cinnamomensis* ATCC 15413? Both ATCC 12308 and ATCC 15413 produced both whole-broth antibiotic and methanol soluble antibiotic activities in the Stark et al. (1968) production medium. When concentrated methanol extracts of ATCC 15413 cultures grown in medium 2 were subjected to thin-layer chromatography on silica gel, the major peaks corresponded to those produced by monensin Na or  $\text{CHCl}_3$  extracts of rumensin (Table 2). The reaction of these chromatographic zones to vanillin was similar to that of authentic samples of monensin Na or of extracts of rumensin, which produced two chromatographic zones that turned red following reaction with vanillin. Typically, the methanol extracts of ATCC 12308 cultures contained two to three solutes with  $R_F$  values of 0.08–0.19, 0.36–0.45, and 0.93–0.97 (minor component). The second solute migrated, typically, just below the lower chromatographic zone produced by monensin Na; but unlike monensin or extracts from ATCC 15413 cultures, it produced a blue reaction color with vanillin rather than a red color. Bioassay of the chromatogram indicated that both the lower purple zone and the middle blue zone were highly active against *B. megaterium*. The tentative conclusion of these studies was that the two strains of *S. cinnamomensis* do not produce the same antibiotics, though both produced methanol soluble antibiotics, which based on their solubility and reaction with vanillin were presumptively of the polyether type.

The methanol extract of ATCC 12308 cultures had antimicrobial activity against *Alcaligenes faecalis* ATCC 19018, *Bacillus megaterium* ATCC 14581, *Pseudomonas aeruginosa*, *Pseudomonas maltophilia* ATCC 13048, *Staphylococcus aureus*, and *Streptococcus faecalis* ATCC 19433. The crude methanol extract was inactive against *Acinetobacter lwoffii* ATCC 7976, *Bacillus cereus* ATCC 14579, *Bacillus subtilis* W23, *Bordetella bronchicamis* ATCC 10580, *Enterobacter aerogenes* ATCC 13048, *Escherichia coli* K-12, *Flavobacterium meningosepticum*, *Moraxella osloensis* ATCC 19976, *Pseudomonas aeruginosa* ATCC 10145, *Sarcina lutea*, *Serratia marcescens* ATCC 13880, *Shigella sonnei* ATCC 10580, and *Staphylococcus epidermidis* ATCC 14990. The monensin Na control was active against *Alcaligenes faecalis* ATCC 19018, all of the *Bacillus* species, *Moraxella osloensis* ATCC 19976, and *Pseudomonas aeruginosa*, *Sarcina lutea*, and *Staphylococcus aureus*. These results differ from those reported

TABLE 2. Results of silica gel thin-layer chromatography of polyether antibiotics and extracts of cultures of *S. cinnamonensis* ATCC 15413 and *S. cinnamonensis* ATCC 12308

Sample	Amount	Vanillin reaction color	Ehrlich reaction	Zone cm	R <sub>F</sub>
CH <sub>3</sub> OH EXT ATCC 15413 Medium 1	10 μl			0.6-3.1 6.1-7.4	0.11 0.51
CH <sub>3</sub> OH EXT ATCC 15413 Medium 2	10 μl	red red		0.5-4.0 5.3-5.7 6.7-7.5 12.2-12.8	0.17 0.42 0.55 0.96
CH <sub>3</sub> OH EXT ATCC 12308 Whole broth Medium 1	10 μl	purple blue		1.4-2.9 5.1-5.9	0.16 0.42
CH <sub>3</sub> OH EXT ATCC 12308 Whole broth Medium 2	5 μl	purple blue	purple blue red lt blue	1.9-3.0 5.0-6.5 5.8-6.3 7.0-7.8	0.19 0.44 0.47 0.57
CHCl <sub>3</sub> EXT ATCC 12308 Mycelium	5 μl	trace blue lt blue lt green blue/grey	purple lt pink blue white grey/green white	2.0-2.8 4.6-6.5 7.0-7.6 7.8-9.6 8.8-10.1 10.9-12.6	0.18 0.42 0.56 0.66 0.73 0.90
CH <sub>3</sub> OH EXT ATCC 12308 CHCl <sub>3</sub> EXT Mycelium	5 μl	blue purple blue lt purple	purple purple pink pink	0.3-0.6 1.8-2.7 5.2-6.5 6.7-7.7	0.04 0.17 0.45 0.55
CHCl <sub>3</sub> EXT Rumensin	13.7 μg	red red	green green	5.6-7.1 7.1-8.8	0.49 0.61
Monensin Na	5.2 μg	red red		5.4-5.8 6.8-7.8	0.43 0.57
A 204 A	5.8 μg	purple		9.6-10.8	0.79
Dianemycin	17.1 μg	lavender lavender red		2.8-3.9 5.5-6.9 7.3-7.8	0.26 0.48 0.59
Lasalocid Na	19.9 μg	blue-grey		10.6-11.7	0.86
X-205 Na	8.6 μg	violet		7.4-8.3	0.61
Nigericin	17.8 μg	purple purple		5.0-6.2 6.5-7.0	0.43 0.52

by Haney and Hoehn (1968) in that the methanol extract was active against gram-negative bacteria as well as gram-positive species. However, until the antibiotic(s) responsible for this activity are purified these results can only be considered as tentative.

Based on the biological activities of the extracts, the mycelial antibiotic(s) produced by ATCC 12308 were not soluble in water but were soluble in the organic

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solvents in order of decreasing solubility: methanol, *n*-butanol, chloroform, acetone, dichlorethane. The antimicrobial component of the methanol extract retained 96% of its activity after the broth was heated to either 50 or 75 C and 92% after being heated to 100 C. Acidifying the broth to pH 2.0 before extraction with methanol resulted in the extract having a slightly greater activity than when the broth was extracted at pH 8. When NaOH was added to the fermentation liquor adjusting to pH 10.0 before extraction with methanol, there was 8% lower activity in the resulting extract. Haney and Hoehn (1968) reported that monensin was stable as the sodium salt but degraded rapidly under acidic conditions. Temperature stability values were not reported.

Comparison of the methanol extracts by paper and thin-layer chromatography (Table 2) of fermentation broth and mycelium from ATCC 12308 and ATCC 15413 cultures combined with the color reactions of the chromatographic zones indicates that the two strains do not produce identical methanol soluble substances. The results of silica gel thin-layer chromatography are reported in Table 2. Of the authentic samples of polyether antibiotics, nigericin was the closest in  $R_F$  values and color reaction with vanillin to those of the methanol extracts of ATCC 12308. Bioassay of the methanol soluble components initially failed to resolve any of the zones, that is, inhibition of *B. megaterium* occurred from the spot of application to the solvent front on silica-gel thin-layer chromatograms. Later bioassays of sufficiently diluted samples obtained by extracting the mycelium of ATCC 12308 with chloroform and then by methanol indicated that the bioactive components of ATCC 12308 migrated on Whatman No. 3MM chromatography paper when developed by ethyl acetate at an average  $R_F$  value of 0.11 and 0.46 whereas the rumensin bioinhibition occurred at an average  $R_F$  value of 0.84. The results obtained with bioassay of silica gel thin-layer chromatograms of either of these extracts of ATCC 12308 produced inhibition at an average  $R_F$  value of 0.12 (0-0.23) and rumensin  $R_F$  0.52 (0.30-0.72). Thus the bioactive component(s) of the methanol extracts of the ATCC 12308 culture is different from that of rumensin.

By varying the inoculum level (0.5, 1.0, 2.0, 5.0, and 10.0% v/v) and measuring the production of both whole-broth and methanol soluble antibiotic activities in the Stark et al. (1968) production medium, an inoculum of 2.0% was optimum for the production of antibiotic activities by ATCC 12308 (data not shown).

The results of a study in which components of medium 3 were sequentially eliminated are presented in Table 3. The deletion of Soytone from the medium strongly enhanced production of methanol extractable antibiotic activity by ATCC 12308 even though the packed cell volume was reduced by 76%. The deletion of any of the minerals but especially  $\text{CaCO}_3$ ,  $\text{K}_2\text{HPO}_4$ , and  $\text{Fe}_2(\text{SO}_4)_3$  inhibited both growth and production of methanol extractable antibiotic(s). The elimination of glucose enhanced production of methanol extractable activity whereas the elimination of dextrin from the medium resulted in neither whole-broth nor methanol extractable activity being produced. The deletion of yeast extracts from medium 3 resulted in enhanced whole broth activity by ATCC 12308. The results indicated that the whole broth and methanol soluble activities are affected differently by the medium components and, thus, there are presumably antibiotics other than the polyether antibiotics present in the whole broth.

Stark et al. (1968) reported a marked increase in the titer of monensin when the refined soybean oil was supplemented by the addition of methyl oleate. In this

TABLE 3. *Effect of deletion of components of medium 3 on growth, pH, whole-broth, and methanol-extractable antibiotic activities produced by ATCC 12308*

Component eliminated	Final pH	Packed cell volume %	Whole-broth relative activity	Methanol extractable relative activity
None	8.5	16.5	1.0	1.0
Glucose	8.8	10.0	1.1	1.3
Soytone	7.0	4.0	2.6	1.9
Dextrin	9.0	10.5	0.0	0.0
Yeast extract	7.0	12.5	1.7	0.9
CaCO <sub>3</sub>	8.2	13.0	0.4	0.6
KCl	8.6	7.2	0.8	0.7
K <sub>2</sub> HPO <sub>4</sub>	8.2	9.5	0.9	0.6
MnCl <sub>2</sub>	8.3	10.5	1.0	0.9
Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub>	8.4	9.8	0.8	0.7

study the addition of soybean oil or a fatty acid ester supplementation of a basal medium was investigated with differing results (Table 4). Methyl oleate, at least by itself, did not increase the production of methanol extractable antibiotics. The supplementation of the basal medium in which ATCC 12308 was growing with ethyl acetate was markedly stimulatory to production of the presumably polyether type antibiotics in the methanol extracts. Acetate was not especially stimulatory to the production of monensin.

TABLE 4. *Effect of supplementation of medium 3 with 1.0% soybean oil or fatty acid esters on antibiotic production by ATCC 12308*

Supplement added	Final pH	Packed cell volume %	Whole-broth relative activity	Methanol extractable relative activity
None	8.4	9.0	1.0	1.0
Soybean oil	8.0	18.0	1.0	0.6
Ethyl acetate	8.5	10.4	0.8	2.5
Methyl decanoate	7.7	1.8	0.0	0.0
Methyl formate	5.2	1.8	0.0	0.0
Methyl hexanoate	8.7	6.6	1.1	1.1
Methyl laurate	8.2	9.4	0.2	0.0
Methyl myristate	7.9	11.0	0.6	0.2
Methyl octanoate	6.9	6.4	0.6	0.0
Methyl oleate	7.7	13.0	1.6	0.6
Methyl palmitate	7.6	13.5	1.1	0.5
Methyl propionate	8.4	9.2	0.8	0.4
Methyl stearate	7.7	16.5	0.7	0.6

## DISCUSSION

The chromatographic studies described above indicated that *S. cinnamomensis* ATCC 12308 does not produce the two major components of monensin Na that are produced by *S. cinnamomensis* ATCC 15413. Under the conditions of our ex-

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periments ATCC 15413 did not produce the methanol extractable substances synthesized by ATCC 12308. This does not indicate that ATCC 15413 cannot produce these compounds, only that it did not do so under the conditions we employed. The two major components in the methanol extracts of ATCC 12308 cultures appear to be similar to nigericin.

The results of the physiological study of *S. cinnamomensis* ATCC 12308 were markedly different from those reported by Stark et al. (1968) for *S. cinnamomensis* ATCC 15413. The production of monensin was not reported to be sensitive to substitutions or variations of concentrations of glucose in the fermentation medium. The elimination of dextrin in this study markedly lowered antibiotic titers. The deletion of glucose actually produced a small increase of antibiotic titer when dextrin remained. The increase in methanol extractable antibiotic titer when Soytone was eliminated also differs from the results reported for ATCC 15413.

Both cultures of *S. cinnamomensis* apparently are sensitive to the presence of minerals such as iron and calcium for the production of polyether type antibiotics. The requirements of ATCC 12308 were not quantified. Stark et al. (1968) reported an optimum concentration of 0.3 g/liter of  $\text{Fe}_2(\text{SO}_4)_3$  for the production of monensin by ATCC 15413.

The observation that ethyl acetate but not methyl oleate was stimulatory to the production of polyether type antibiotics by ATCC 12308 differs markedly from the results reported by Stark et al. (1968) for ATCC 15413.

The results presented in this study establish that *S. cinnamomensis* ATCC 12308 and *S. cinnamomensis* ATCC 15413 are physiologically different.

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