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Characterization of the Antitumor Glucan Obtained from Liquid-Cultured *Grifola frondosa*

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Chemical characterization of the extracts, and purification and structural characterization of an antitumor glucan obtained from liquid-cultured mycelium of *Grifola frondosa* are described in this paper. The mycelium (13 g dry weight/l culture) was extracted successively with hot water (LMHW, 14.9 g/100 g mycelium), cold alkali (LMCA, 6.3 g/100 g mycelium), and hot alkali (LMHA, 4.5 g/100 g mycelium). Each extract was dialyzed and then the polysaccharide fraction was precipitated with ethanol. Another portion of the mycelium was incubated with a buffer composed of glucose (5.0%) and citric acid, pH 4.5, for 3 d and the supernatant was obtained (LELFD, 50 g/100 g mycelium). The broth was dialyzed and the nondialyzable fraction was precipitated with ethanol (LLFD, 1.3 g/l culture). All of the above fractions were composed of glucose as the major carbohydrate. Except for LMHW, all fractions showed antitumor activity against sarcoma 180 cells in ICR mice. The activity was the strongest in the LELFD fraction. Antitumor glucan (grifolan LE) was purified from LELFD by diethylaminoethyl-Sephadex A-25 chromatography and ethanol precipitation. Grifolan LE gave M_r of more than 5×10^6 (8 M urea/0.2 N NaOH), $[\alpha]_D$ $-6°$—$-9°$ ($c = 0.1\%$; 0.3 N NaOH), and showed metachromasy coupled with Congo red. By carbon-13 nuclear magnetic resonance spectra and methylation analyses, grifolan LE was found to be a branched β-1,3-glucan containing a branch at C-6 of every three main chain glucosyl units.

Keywords——*Grifola frondosa*, antitumor glucan, fungi, Basidiomycotina, β-1,3-glucan, mycelium

Fruit body and liquid-cultured mycelium of fungi belonging to Basidiomycotina have been reported to contain useful antitumor polysaccharides.[1] Antitumor polysaccharides have been obtained from various fractions, such as the hot water extract of the fruit body ("lentinan"),[1b] the hot water extract of liquid-cultured mycelium ("krestin"),[1c] and the extracellular polysaccharide of liquid-cultured mycelium ("schizophyllan").[1d] Recently, we have studied the structure and antitumor activity of the hot water, cold alkali, and hot alkali extracts of the fruit body and the matted mycelium of *Grifola frondosa*.[2] These fractions contained neutral D-glucans(α-1,4- and β-1,3-) and acidic glucans(β-1,6- and β-1,3-). The antitumor principle was demonstrated to be a β-1,3-glucan possessing a branch at C-6 of every three main chain glucosyl units. The present paper is concerned with the structural characterization of an antitumor glucan from liquid-cultured *G. frondosa*.

Materials and Methods

Cultivation——Cultivation was performed according to the procedure described previously.[3] Briefly, *Grifola frondosa* var. *Tokachiana* was grown in 6000 ml (100 ml each) of medium containing glucose (2.0%), polypeptone (0.6%), cane sugar (2.0%) and soybean oil (0.1%), pH 4.5, with reciprocal shaking at 25 °C for 14 d. By filtration, 75 g (dry weight) of mycelium cake and the filtrate were obtained. The filtrate was dialyzed against water and the non-dialyzable fraction was precipitated by adding 4 volumes of ethanol (LLFD, 1.3 g/l culture).

Extraction of Hot Water (LMHW), Cold Alkali (LMCA), and Hot Alkali (LMHA) Extracts from the Mycelium——Extraction of polysaccharide fractions was performed by essentially the same procedure as used for the fruit body.[2] Briefly, the lyophilized mycelium (65 g) was defatted by refluxing with aqueous 80% ethanol, acetone and ether. The defatted mycelium was extracted with water in an autoclave for 1 h (121 °C, 7 times). The combined extracts were dialyzed and then precipitated with 4 vols. of ethanol (LMHW 9.7 g). The residual mycelium was extracted with 5% urea containing 10% NaOH aqueous solution at 4 °C for 24 h (3 times) and then at 60 °C for 1 h (3 times). The cold and hot alkali extracts were neutralized with acetic acid and then dialyzed. The non-dialyzable fractions were precipitated with 4 vols. of ethanol (LMCA 4.1 g, LMHA 2.9 g).

Preparation of the Extracellular Polysaccharide——The lyophilized mycelium (13 g) was suspended in a buffer containing glucose (5.0%) and citric acid, pH 4.5. The suspension was incubated at 25 °C for 3 d. The resulting supernatant was obtained by centrifugation. These procedures were repeated 2 to 3 times. The combined supernatant was dialyzed against water and the non-dialyzable fraction was precipitated with ethanol (LELFD 6.3 g).

Purification of Grifolan LE——LELFD (50 mg) dissolved in 8 M urea was applied to a column of DEAE-Sephadex A-25 (HCO_3^-) (20 ml). The column was eluted with 8 M urea and then with a linear gradient from 0 to 1 M NH_4HCO_3 containing 8 M urea. Finally, the column was eluted with 2 M NaCl containing 8 M urea. After extensive dialysis, the fraction eluted with 8 M urea was precipitated with 1.5 vols of ethanol (grifolan LE).

Other Methods——Other methods (quantitative determination, antitumor assay, carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy, and physicochemical analyses) were performed as described previously.[2]

Results

Chemical Properties of Fractions Obtained from the Liquid-Cultured *Grifola frondosa*

Polysaccharide fractions were extracted from the liquid-cultured mycelium with hot water, cold alkali, and hot alkali by procedures similar to those used in the case of the fruit body.[2] Polysaccharide fraction from the culture fluid was also obtained. Some of its properties are listed in Table I. The major component sugar was glucose in all fractions. Yields of LMHW, LMCA, and LMHA were similar to those of the fruit body and the matted mycelium. Fig. 1a—c shows the ^{13}C-NMR spectra of these fractions. From a comparison of the intensities of α (101 ppm) and β (104 ppm) anomeric signals, LMHW, LMCA, and LMHA contained a large amount of α-glucans. This is in contrast to the ratio of α- and β-glucans in the fractions obtained from the fruit body and the matted mycelium, in which β-glucan contents were higher than the α-glucan contents. Figure 1g shows the ^{13}C-NMR spectrum of the mycelium as an aqueous suspension. The signal of α-glucan (101 ppm) is stronger than that of β-glucan (104 ppm), and the ratio of α- and β-glucan in this spectrum is consistent with the results for the extracts described above. Figure 1d,e shows the ^{13}C-NMR spectrum of LLFD in 0.5 or 0.2 N NaOH solution. In 0.2 N NaOH, LLFD showed broader signals than others. This suggests that the conformational rigidity of LLFD is greater.

Previously, Oikawa *et al.* reported a method for the production of extracellular polysaccharides (LELFD) from the mycelium of *G. frondosa*.[3] We next examined the physicochemical properties of LELFD (Table I). The yield of LELFD was about 50 g from 100 g of the lyophilized mycelium. Chemical analysis showed that LELFD is composed

TABLE I. Some Properties of Polysaccharide Fractions Obtained from Liquid-Cultured Mycelium of *Grifola frondosa*[a]

Fraction	Yield[b]	Carbohydrate (%)	Protein (%)	Component sugars
LMHW	14.9	73	9	Glc
LMCA	6.3	41	28	Glc
LMHA	4.5	47	26	Man/Glc (0.05/1)
LLFD	10.8	53	2	Man/Glc (0.16/1)
LELFD	48.5	85	9	Xyl/Man/Glc (0.01/0.03/1)

a) Abbreviations used are LMHW, hot water extract; LMCA, cold alkali extract; LMHA, hot alkali extract; LLFD, cultured filtrate; LELFD, extracellular polysaccharide fraction obtained by incubation of mycelium in buffer. *b*) From 100 g of the lyophilized mycelium.

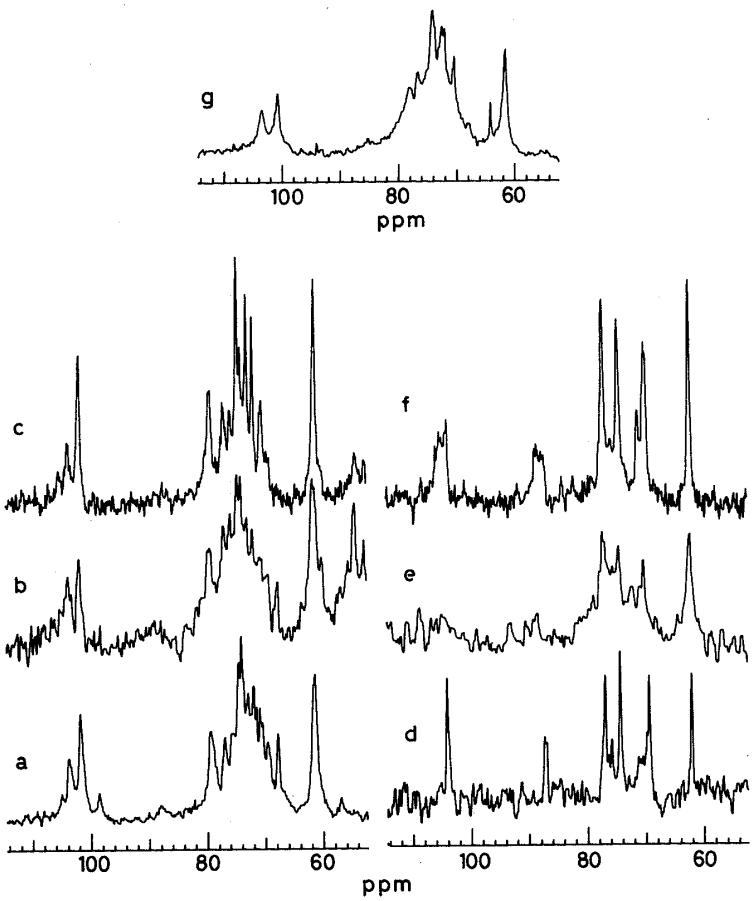

Fig. 1. ^{13}C-NMR Spectra of Polysaccharide Fractions Obtained from Liquid-Cultured *G. frondosa*

a, b, c: ^{13}C-NMR spectra of LMHW, LMCA, and LMHA, respectively in 0.2 N NaOH. d, e: ^{13}C-NMR spectra of LLFD in 0.5 N and 0.2 N NaOH, respectively. f: ^{13}C-NMR spectrum of LELFD in 0.3 N NaOH. g: ^{13}C-NMR spectrum of liquid-cultured mycelium of *G. frondosa* as aqueous suspension in H_2O.

mainly of carbohydrates (85%). The main component sugar was glucose as determined by gas liquid chromatography. From the results of ^{13}C-NMR spectroscopy (Fig. 1f), in contrast to LMHW, LMCA, and LMHA, LELFD showed only β-glucan signals in the anomeric region of the spectrum. Strong signals at 88 ppm attributable to substituted C-3 of β-glucoside were seen. In methylation analysis, 2,3,4,6-tetra-*O*-Me, 2,4,6-tri-*O*-Me, and 2,4-di-*O*-Me-glucose residues were detected in a molar ratio of 1.0 : 1.7 : 1.0. In some lots, this fraction contained a trace amount of 3,4,6-tri-*O*-Me or 2,6-di-*O*-Me-glucose derivative.

Antitumor Activity against Solid Form of Sarcoma-180 of Fractions Obtained from the Liquid Culture of *G. frondosa*

The antitumor activities of the five fractions (LMHW, LMCA, LMHA, LLFD, and LELFD) obtained from the liquid culture described above were examined by using the assay system of Sarcoma-180 solid tumors in ICR mice (Table II). In the previous paper, it was shown that the hot water, cold alkali, and hot alkali extracts of the fruit body and the matted mycelium showed potent antitumor activity.[2] In contrast, the hot water extract (LMHW) showed no antitumor activity under these conditions and at the same concentration. Further, in the cases of the fruit body and the matted mycelium, the cold alkali and hot alkali extracts showed antitumor activity at doses of lower than 40 μg/mouse/d ($\times$ 10 d).[2] However, LMCA and LMHA showed antitumor activity at doses of more than 400 μg/mouse/d ($\times$ 5 d). LLFD showed stronger activity than LMHW, LMCA, or LMHA.

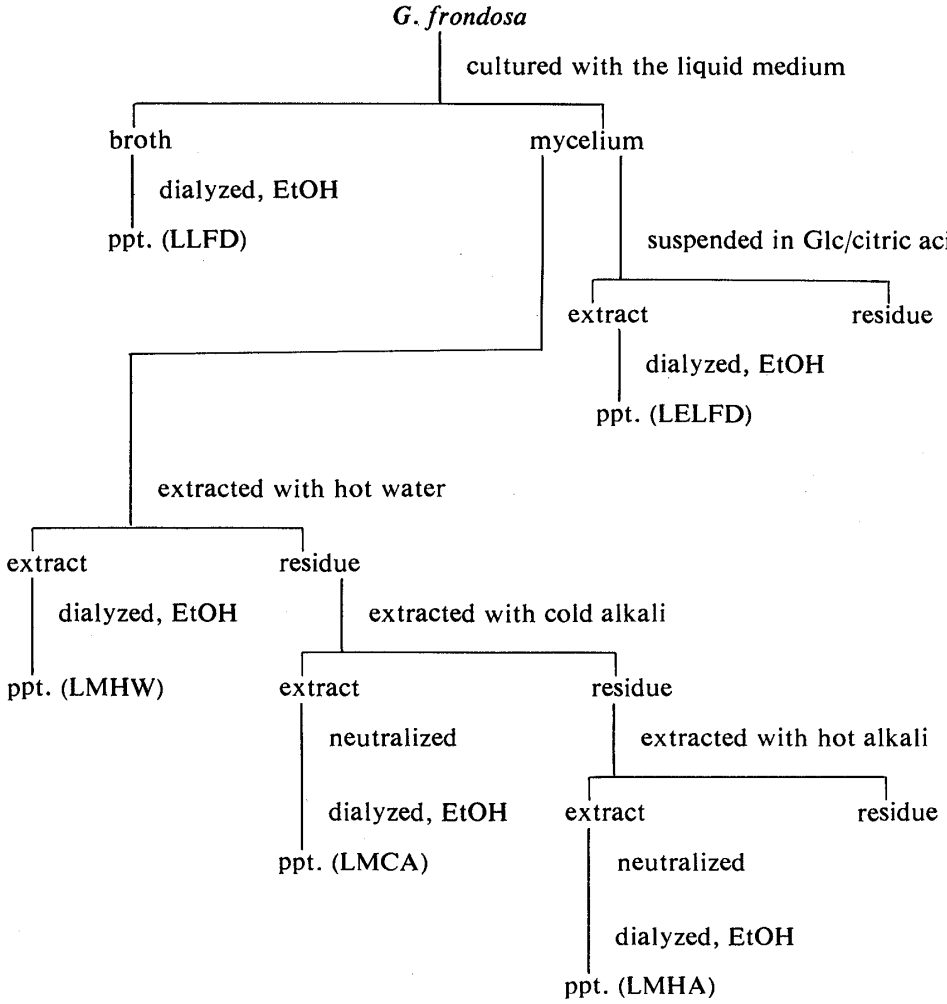

Chart 1. Isolation of Polysaccharide Fractions from Liquid-Cultured *G. frondosa*

The activities of the extracellular polysaccharide fractions prepared by first (LELFD-1) and second (LELFD-2) incubations were compared. Both fractions showed similar activities. The activity of LELFD was the strongest among the five fractions and was comparable to that of the fruit body or the matted mycelium.

Purification of an Antitumor Glucan from LELFD

We used the LELFD fraction as the starting material to isolate an antitumor glucan from the liquid culture. In the cases of the fruit body and the matted mycelium, it has been clarified that the extracts contain both neutral and acidic glucans. As shown in Fig. 2b, LELFD contained only neutral glucan fractions (LELFD-1). To clarify why LELFD contained no acidic glucans, the elution profile was compared with that of LMCA prepared by the alkaline extraction method. As shown in Fig. 2a, LMCA contained only a small amount of acidic fractions compared with those of the fruit body and the matted mycelium. It appears that the acidic glucan content of the liquid-cultured mycelium is lower than those of the fruit body and the matted mycelium.

Since comparison of the elution profiles of LELFD and LMCA indicated that the acidic glucan and the protein contents of these fractions were quite different, it was concluded that the preparation method from the extracellular polysaccharides was better for obtaining the antitumor glucan than other methods.

LELFD-1 was further purified by ethanol precipitation. Most of the glucan (64% of that

Table II. Antitumor Activity against Sarcoma 180 of Fractions Obtained from the Liquid Culture of *Grifola frondosa*[a]

Fraction	Dose ($\mu g \times 5$)	Tumor weight[c] (g, mean ± S.D.)	Inhibition[b] ratio (%)	Complete regression[b]
(Experiment 1)				
LMHW	400	4.0±2.3	24	1/10
	4000	5.1±5.0	4	0/10
LMCA	400	1.2±1.2[d]	77	3/10
	4000	0[f]	100	9/9
LMHA	400	1.6±1.6[d]	71	3/10
	4000	0.1±0.1[e]	99	6/10
LLFD	400	0.9±2.0[d]	83	4/10
	4000	<0.1[e]	>99	7/9
Control	—	5.3±4.8	0	1/12
(Experiment 2)				
LLFD	100	2.3±4.4[f]	81	0/10
	1000	1.3±1.7[f]	89	3/10
LELFD-1	100	<0.1[f]	>99	3/9
	1000	5.7±6.7[d]	53	1/10
LELFD-2	100	0.6±1.8[f]	95	4/10
	1000	6.8±5.4[d]	43	0/10
Control	—	12.0±4.3	0	0/16

a) Sarcoma 180 tumor cells (5×10^6) were inoculated subcutaneously (day 0). Each sample was administered as saline solution by intraperitoneal injection on days 1, 3, 5, 7, and 9. *b*) Inhibition and complete regression were determined on day 35 after tumor inoculation. *c*) The significance of differences was evaluated according to Student's *t*-test. Significant difference from control (*d*) $p<0.05$, *e*) $p<0.01$, *f*) $p<0.001$).

Table III. Antitumor Activity of Grifolan LE[a]

	Dose ($\mu g \times 5$)	Tumor weight[b] (g, mean ± S.D.)	Inhibition ratio (%)	Complete regression
Grifolan LE	4	5.0±2.4	7	0/10
	20	3.4±3.5	38	2/10
	100	<0.1[c]	>99	9/10
	200	<0.1[c]	>99	5/10
Nil		5.4±3.5	0	0/10

a) Experimental conditions were the same as in Table II. *b*) The significance of differences was evaluated according to Student's *t*-test. Significant difference from control (*c*) $p<0.001$).

in LELFD-1; carbohydrate 84—91%, protein 0.3—1.8%) was precipitated by the addition of 1.5 volumes of ethanol. After hydrolysis with 1 N trifluoroacetic acid at 100 °C for 5 h, only glucose was detected as a component sugar. As shown in Table III, this glucan showed potent antitumor activity. The optimum dose was similar to that of the glucans obtained from the fruit body and the matted mycelium. This glucan has been named "grifolan LE."

Physicochemical Properties of Grifolan LE

In Sepharose CL-4B chromatography with 0.2 M NaOH–8 M urea, most of the glucan was eluted at the void volume. This fact suggests that the molecular weight of this glucan was more than 5×10^6. The glucan showed $[\alpha]_D$ −6—−9° ($c=0.1\%$; 0.3 N NaOH). The ^{13}C-NMR spectrum of the glucan in DMSO-d_6 showed signals quite similar to those of scleroglucan and the glucans obtained from the fruit body and the matted mycelium of *G. frondosa* (Fig. 3).

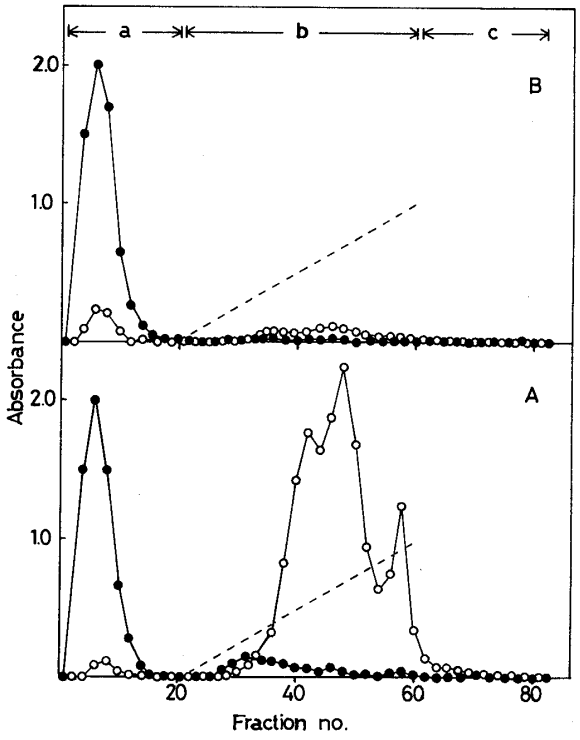

Fig. 2. Elution Profiles of LMCA and LELFD from a Column of DEAE-Sephadex A-25 (HCO_3^-)

The column (20 ml) was equilibrated with 8 M urea, and 50 mg of LMCA (A) or LELFD (B) was applied. After being washed with 8 M urea (a), the column was eluted with a linear gradient of 0 to 1 M NH_4HCO_3 containing 8 M urea (b), and then with 2 M NaCl containing 8 M urea (c). Fractions of 2.8 ml were collected and carbohydrate (—●—) and protein (—○—) were monitored by the phenol-H_2SO_4 method and by ultraviolet absorption measurement, respectively.

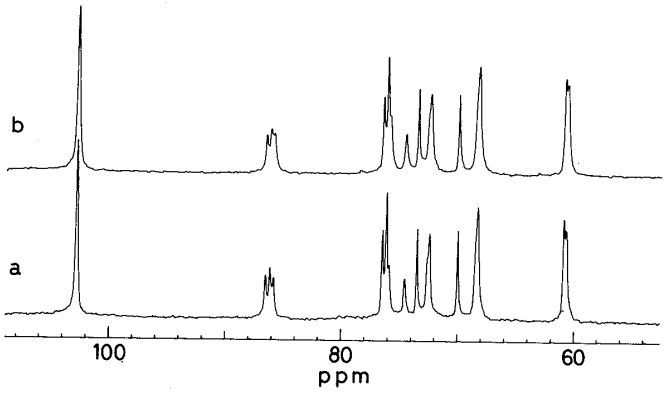

Fig. 3. ^{13}C-NMR Spectrum of Grifolan LE in DMSO-d_6 at 60 °C

a: grifolan NMF-5N (obtained from the matted mycelium of *G. frondosa*). b: grifolan LE.

This glucan showed metachromasy with Congo red (510 nm at 0.1 N NaOH). Further, in methylation analysis, 2,3,4,6-tetra-*O*-Me-, 2,4,6-tri-*O*-Me-, and 2,4-di-*O*-Me-glucose were detected in a molar ratio of 1.0 : 1.8 : 1.0. These results suggest that the glucan is a β-1,3-glucan possessing a branch at position 6 of every three main chain glucosyl units.

Discussion

It was previously shown that the fruit body and the matted mycelium contain both neutral and acidic glucans, and the major glucan showing antitumor activity is a β-1,3-glucan possessing a branch at position 6 of every three main chain glucosyl units. Thus, polysaccharides in the liquid-cultured mycelium showed the following characteristics. (1) The mycelium contained both α- and β-glucans, but contained only a small amount of acidic glucans. (2) The extractability of the β-glucan was less than that from the fruit body or the matted mycelium. (3) Antitumor polysaccharide obtained from the liquid-cultured mycelium had a structure quite similar to that of the fruit body.

The preparation of β-1,3-glucan from the mycelium after incubation with glucose is quite

simple and useful. This procedure is milder than those used to obtain other antitumor glucans, such as hot water extraction (PSK, lentinan)[1a] or alkali extraction (grifolan).[2] Further, this preparation contained only a small amount of materials originating from the broth. Recently, we also obtained successful results by using a jar fermenter for obtaining mycelium instead of shaking-culture flasks. Therefore, this is a rather easy method to prepare the antitumor glucan, and should be suitable for large-scale use.

Glucans of similar structure have been found in fungi, and some of these polysaccharides, such as lentinan (from *L. edodes*)[1a] and schizophyllan (from *S. commune*)[1d] are now used clinically. However, the M_r of grifolan is larger than those of the above polysaccharides. It would be interesting to study the mechanism of antitumor activity comparatively.

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