Carbohydrate Research, 74 (1979) 217–225

STRUCTURE OF THE WATER-INSOLUBLE α-D-GLUCAN OF *Streptococcus salivarius* HHT

YOICHI TSUMURAYA AND AKIRA MISAKI*

Department of Food and Nutrition, Faculty of Science of Living, Osaka City University, Sugimoto-cho, Sumiyoshi, Osaka 558 (Japan)

(Received September 26th, 1978; accepted for publication in revised form, November 20th, 1978)

ABSTRACT

Water-insoluble, non-adherent α-D-glucans have been obtained from *Streptococcus salivarius* HHT under two sets of conditions: from a growing culture, or synthesized enzymically by using a glucosyltransferase. In the former case, the glucan ($[\alpha]_D$ +197°) was shown by methylation analysis to have a slightly branched structure containing a relatively high proportion (80%) of (1→3)-α-D-glucosidic linkages, together with small proportions of (1→6)- and (1→4)-α-D-glucosidic linkages. The enzymically synthesized glucan had a much less-branched structure, containing 88% of (1→3)-α-D-glucosidic linkages. Both glucans, on Smith degradation (sequential periodate oxidation, borohydride reduction, and mild acid hydrolysis), gave linear, (1→3)-α-D-glucosidic polysaccharides (yields, 82–90%) that constitute the backbone chains. The presence of small proportions of glycerol, erythritol, 1-*O*-α-D-glucosyl-D-glycerol, and also 2-*O*-α-D-glucosyl-D-erythritol in the products of Smith degradation suggests that the short side-chains are attached to the backbone chain by (1→4)-, (1→6)-, and (1→3)-α-D-glucosidic linkages.

INTRODUCTION

Water-insoluble α-D-glucans elaborated by such oral Streptococci as, *e.g.*, *Streptococcus mutans*[1], *S. sanguis*[2], and *S. salivarius*[3], have recently been extensively studied in relation to the formation of dental plaque and subsequent development of caries. These insoluble glucans are known to contain high proportions of (1→3)-α-D-glucosidic linkages. In the previous paper[4], we showed that the insoluble α-D-glucan of the cariogenic *S. mutans* OMZ 176 is made up of an α-(1→3)-linked backbone chain to which short side-chains of α-(1→6)-linked D-glucosyl residues are attached. In a series of chemical and enzymic studies of various types of polysaccharides of oral Streptococci and related microorganisms, we became aware that a weakly cariogenic strain of *S. salivarius* HHT that produces a levan type of fructan also produces a non-adherent, water-insoluble α-D-glucan[5]. The present paper is concerned

*To whom requests for reprints should be sent.

with the detailed structural features of the less-branched glucans synthesized by a growing culture of *S. salivarius* HHT, and also by its glucosyltransferase.

RESULTS AND DISCUSSION

The extracellular, insoluble glucan was obtained from a culture of *S. salivarius* HHT, grown in a medium containing 5% of sucrose and Trypticase soy-broth. In order to remove the water-soluble levan, the cultural broth was centrifuged, and the insoluble glucan obtained by careful extraction of the insoluble residue with M potassium hydroxide. Thus, from 4 L of broth, 0.45 g of the purified glucan was obtained, together with 12.3 g of levan. The enzymically synthesized glucan was obtained by incubation of the glucosyltransferase fraction, precipitated from the 1.7-L culture-filtrate of the microorganism by 50% saturation with ammonium sulfate, with 10% sucrose at pH 6.8. The insoluble glucan was collected by centrifugation, and purified as for the extracellular glucan. This procedure gave soluble levan and insoluble glucan (ratio, 3.1 g:250 mg). Both glucan preparations were essentially free from protein, and yielded only glucose (98–100%) on hydrolysis with acid. The homogeneity of the extracellular glucan was assessed by ultracentrifugal analysis in M sodium hydroxide solution, which gave a single, symmetrical peak ($s_{20,w}$ 2.02 *S*). The high optical rotation, $[\alpha]_D$ +197° (*c* 0.45, M sodium hydroxide), and the characteristic absorbance at 840 cm^{-1} in the i.r. spectrum were indicative of the α-D-glucosidic linkage.

To examine the linkage sequence, the glucans were methylated by the method of Hakomori[6]. Both methylated glucans were hydrolyzed by acid, and the methylated sugar fragments were examined by gas–liquid chromatography (g.l.c.) as their corresponding alditol acetate derivatives. The identities and molar proportions of the D-glucosidic linkages in both glucans are listed in Table I. The methylation data indicate that both the extracellular and enzymically synthesized, insoluble glucan

TABLE I

MOLAR RATIOS OF HYDROLYSIS PRODUCTS FROM METHYLATED EXTRACELLULAR AND ENZYMICALLY SYNTHESIZED, WATER-INSOLUBLE GLUCANS

O-Methyl-D-glucose	*Linkage indicated*	*Molar percent*	
		Extracellular glucan	*Enzymically synthesized glucan*
2,3,4,6-Tetra-	Glc*p*-(1→	3.5	1.7
2,4,6-Tri-	→3)-Glc*p*-(1→	80.4	87.8
2,3,4-Tri- and 2,3,6-Tri-	→6)-Glc*p*-(1→ and →4)-Glc*p*-(1→	12.2	8.3
2,4-Di-	→6)-, →3)-Glc*p*-(1→	3.9	2.2

contain a very high proportions of (1→3)-α-D-glucosidic linkage, 80.4% in the extracellular, and 87.8% in the enzymically synthesized glucan, together with small proportions of α-(1→4) and α-(1→6) linkages. The formation of a small amount of 2,4-di-*O*-methyl-D-glucose from both glucans indicates that they have slightly branched structures having average repeating-units of 29 for the extracellular, and 59 for the enzymically synthesized glucan. Under the conditions employed for the g.l.c. analyses, 2,3,4- and 2,3,6-tri-*O*-methyl-D-glucose were not readily separated, but the presence of these two tri-*O*-methyl-D-glucoses in both methylated glucans was confirmed by g.l.c. analyses of the methylated sugar components as their corresponding methyl glucosides.

The foregoing methylation result was supported by periodate oxidation, and also by Smith degradation. The extracellular and the enzymically synthesized glucans were each oxidized with 0.08M sodium metaperiodate for 10 days at 25°. At the time of complete oxidation, liberation of formic acid and periodate consumption were 0.09 and 0.35 mol per glucose residue for the extracellular glucan, and 0.05 and 0.20 mol for the enzymically synthesized glucan, respectively. After complete oxidation, both oxidized glucans were reduced by sodium borohydride to their corresponding glucan-polyalcohols. A portion of each glucan-polyalcohol (5 mg) was hydrolyzed completely[7] with 2M sulfuric acid for one h at 100°, and the products were examined by paper chromatography, which revealed the presence of glucose as the major product, together with small proportions of glycerol and erythritol. It is evident that glycerol is derived from the nonreducing terminals and the α-(1→6)-linked D-glucosyl residues, and erythritol from α-(1→4)-linked D-glucose residues, whereas glucose would arise from α-(1→3)-linked D-glucosyl residues and the branch points. The quantitative determinations of the products after complete, acid hydrolysis of the

TABLE II

MOLAR RATIOS OF PRODUCTS FROM SMITH DEGRADATION OF EXTRACELLULAR AND ENZYMICALLY SYNTHESIZED, WATER-INSOLUBLE GLUCANS[a]

Glucan	*Product*	*Molar percent*	
		L.c.[b]	*G.l.c.*[c]
Extracellular	glycerol	10.0	9.5
	erythritol	4.4	4.5
	glucose	85.6	86.0
Enzymically synthesized	glycerol	7.5	5.8
	erythritol	3.2	3.5
	glucose	89.3	90.7

[a]The glucan-polyalcohol was hydrolyzed with 2M sulfuric acid for one h at 100°. [b]Analyzed with a Yanaco Liquid Chromatograph Model L-1030 fitted with a refractive-index detector, on a column of SCX 1001 (6 × 500 mm), with water as carrier, at 25°. [c]Analyzed by gas–liquid chromatography of the alditol acetates, on a column of 3% of ECNSS-M at 100–190° (6°/min).

glucan-polyalcohols derived from both glucans, synthesized by a growing culture and by the glucosyltransferase, were conducted by high-performance liquid chromatography (l.c.) and also by g.l.c. The results summarized in Table II are in good agreement with those expected from the methylation data. Thus, the results of methylation analysis and Smith degradation indicate that the extracellular glucan contains 4.5% of α-(1→4)- and 6% of α-(1→6)-D-glucosidic linkages. Similarly, the enzymically synthesized glucan was shown to contain 3.5% of α-(1→4)- and 4% of α-(1→6)-D-glucosidic linkages.

In order to obtain more knowledge of the sequences of α-(1→3)- and non-(1→3)-linked D-glucosyl residues, the glucan-polyalcohols derived from both glucans were hydrolyzed under mild conditions, using with 50mM sulfuric acid for 20 h at 25° (mild Smith degradation), and the degradation products in the water-soluble and -insoluble fractions were examined. Under these conditions, the yields of water-insoluble, degraded glucans were 82% for the extracellular glucan, and 90% for the enzymically synthesized glucan, respectively. The degraded glucan from the extracellular glucan showed a single, symmetrical peak in ultracentrifugal analysis; its $s_{20,w}$ value of 1.6 *S* was smaller than that of the parent glucan ($s_{20,w}$ = 2.02 *S*). The degraded glucan was methylated, and g.l.c. analysis of the methylated glucose fragments revealed the presence of 2,4,6-tri-*O*-methyl-D-glucose (98%) and traces of 2,3,4,6-tetra-, and 2,4-di-*O*-methyl-D-glucose, the latter probably arising through a slight degree of under-oxidation with periodate. The foregoing methylation data clearly indicate that the degraded glucan, consisting of a long, consecutive sequence of α-(1→3)-linked D-glucosyl residues, constitutes the backbone chain of the parent glucan. This finding confirms the previous conclusion that the water-insoluble glucans elaborated by oral Streptococci contain a (1→3)-α-D-glucan backbone chain[4].

A careful search of the water-soluble products formed simultaneously with

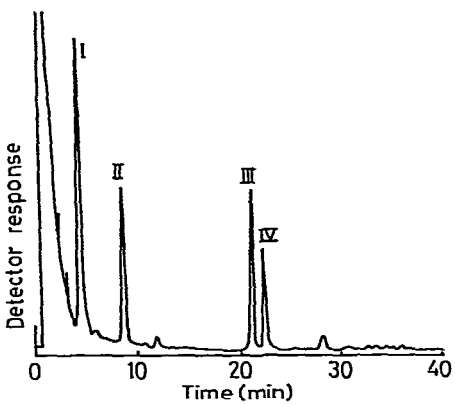

Fig. 1. Gas–liquid chromatographic separation of the soluble fraction of the mild Smith-degradation products from extracellular, water-insoluble glucan. The products were separated as the corresponding trimethylsilyl ethers on a column of OV-17 at 80–320° (6°/min): I, glycerol; II, erythritol; III, 1-*O*-α-D-glucosyl-D-glycerol; IV, 2-*O*-α-D-glucosyl-D-erythritol.

the degraded glucan revealed the presence of glycerol, erythritol, and a component having $R_{G\text{-}c}$ 0.90. Acid hydrolysis of the latter component, as isolated by paper chromatography, gave glycerol, erythritol, and glucose in the molar ratio of 0.75: 0.20:1.00 (g.l.c.), suggesting that it was a mixture of glucosylerythritol and glucosylglycerol. Fig. 1 shows the g.l.c. profile of the soluble fraction from the extracellular glucan, separated as the *O*-trimethylsilyl derivatives. This analysis reveals the presence of glycerol, erythritol, 1-*O*-α-D-glucosylglycerol and 2-*O*-α-D-glucosyl-D-erythritol. The latter two glucosides were identified by comparison of their retention times with those of authentic compounds synthesized by the method of Charlson *et al.*[8]. The soluble fraction from the enzymically synthesized glucan also gave a similar g.l.c. profile. The glycerol must have arisen from the non-reducing terminals and (1→6)-linked D-glucose residues, and erythritol from (1→4)-linked D-glucose residues, probably located in the side chains. The 1-*O*-α-D-glucosylglycerol and 2-*O*-α-D-glucosyl-D-erythritol must have originated from the single, α-(1→3)-linked D-glucose residues, which are adjacent to α-(1→6)- and α-(1→4)-linked D-glucose residues, in the sequences →6) [or →4)]-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→ and →6) [or →4)]-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→, respectively. Assuming that the products found in the soluble fraction all arose from the sequences in the side chains, which are attached to the backbone chain of α-(1→3)-linked D-glucose residues by (1→6)-linkages (Fig. 2), the molar ratios of the D-glucosidic linkages constituting the side chains are summarized in Table III. As the gel-filtration profile on a column of Bio-Gel P-2 showed that the soluble fractions contained neither polysaccharides nor oligosaccharides, consecutive, α-(1→3)-linked D-glucose residues may not be present in the side chains.

From recent studies, it is accepted that most water-insoluble α-D-glucans elaborated by cariogenic Streptococci have branched structures that contain relatively high proportions of (1→3)-α-D-glucosidic linkages. The ratios of α-(1→3)- to (1→6)-linkages appear to vary according to the individual microorganism[9]. Among different α-D-glucans of oral Streptococci, the structure of the α-D-glucan of *S. mutans* OMZ 176, synthesized by its glucosyltransferase, was intensively studied by Guggenheim[1],

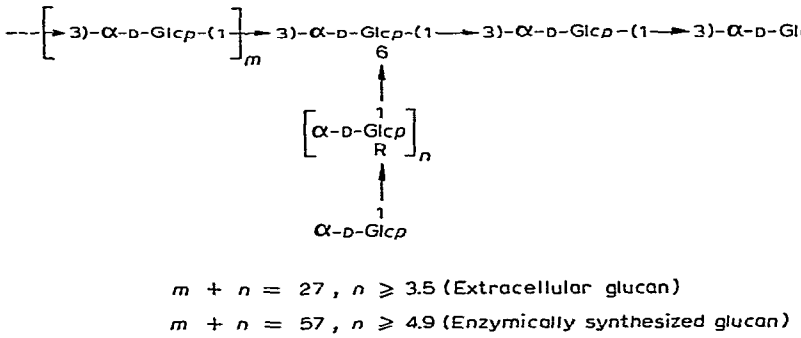

Fig. 2. The possible structure of extracellular and enzymically synthesized, water-insoluble glucans produced by *Streptococcus salivarius* HHT. R denotes O-3, O-4, or O-6.

TABLE III

CONSTITUTION OF THE SIDE CHAINS OF EXTRACELLULAR AND ENZYMICALLY SYNTHESIZED, WATER-INSOLUBLE GLUCANS

Linkage	*Molar ratio*	
	Extracellular glucan	*Enzymically synthesized glucan*
Glc*p*-(1→ and →6)-Glc*p*-(1→	1.00	1.00
→4)-Glc*p*-(1→	0.23	0.27
→3)-Glc*p*-(1→6)-Glc*p*-(1→	0.29	0.22
→3)-Glc*p*-(1→4)-Glc*p*-(1→	0.07	0.05

Ceska *et al.*[2], and Ebisu *et al.*[4]. It was shown that the glucan contains 50% of α-(1→3)-, and 21% of (1→6)-α-D-glucosidic inter-residue linkages, and 14% of branching linkages substituted at O-3 and O-6. Its structure was established by methylation and mild Smith degradation to afford the backbone chain of consecutive, α-(1→3)-linked D-glucose residues, having short, α-(1→6)-linked side-chains[4].

In comparison with the foregoing glucan of *S. mutans* OMZ 176, the insoluble glucan of *S. salivarius* HHT, known as a weak cariogenic strain and producing mainly a levan type of fructan[5], possesses a unique structure consisting of an extremely high proportion of (1→3)-α-D-glucosidic linkages (84–90%, including branching linkages). The side chains comprise a variety of D-glucosidic linkages, namely, (1→6)-, (1→4)- and, unexpectively, (1→3)-linkages, some of which are present in the sequences →3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→, and →3)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→. It should be pointed out that this less-branched D-glucan is water-insoluble, like ordinary streptoccocal α-D-glucans, but it does not possess sticky and adherent characteristics. As α-(1→3)-linked, degraded D-glucan derived from the sticky OMZ 176 insoluble glucan by the mild Smith degradation (which results in cleavage of all side chains) lost its original adherent property[4], the present observations confirm the previous results that the long, α-(1→3)-linked, linear sequence must relate to the insolubility characteristics, and that the presence of many short α-D-(1→6)-linked side-chains may be necessary for the adherent property of the glucan.

As regards the mechanism of biosynthesis of water-insoluble, branched α-D-glucans of oral Streptococci, the backbone chains are made up of long, consecutive α-(1→3)-linked D-glucose residues, and the proportions of (1→3)- and other non-(1→3)-linkages vary with different strains; therefore, it is most likely that the formation or elongation of the (1→3)-linked backbone-chain proceeds, and then an oligosaccharide group containing mainly (1→6)-D-glucosidic linkages may become attached to it to form the branched structures. Such a hypothetical concept is supported by the facts that the insoluble, non-adherent glucan obtained in the present study has a less-branched structure (average repeating unit, 59 for enzymically

synthesized glucan), which consists mainly of α-(1→3)-linked D-glucose residues, and the proportion of (1→3)-linkages (90%, including branch points) in the glucan synthesized by using the crude glucosyltransferase is significantly higher than that formed by the growing culture (84%). Isolation of the glucosyltransferase from *S. salivarius* HHT, and examination of the nature of the insoluble glucan synthesized by the purified enzyme may be necessary to clarify the mechanism of biosynthesis of the water-insoluble glucan.

As already mentioned, *S. salivarius* HHT, used in this study, produces a water-soluble fructan as the major polysaccharide, in addition to the water-insoluble, non-adherent glucan. The previous study showed that the fructan is a levan-type of branched structure, having an average repeating-unit of 9 fructose residues, consisting of (2→6)-β-D-fructofuranosidic linkages; it differed from the cold-water-insoluble fructans of several strains of *S. mutans* that have less branched structures consisting of (2→1)-β-D-fructofuranosidic linkages[5]. The glucosyl- and fructosyl-transferases of *S. salivarius* HHT in the crude enzyme preparation were completely separated from each other by column chromatography on hydroxylapatite (Y. Tsumuraya and A. Misaki, unpublished results). Chemical and biosynthetic studies of the glucan, and also the fructan, synthesized by the purified enzymes, are now in progress.

EXPERIMENTAL

Microorganism. — *Streptococcus salivarius* HHT, which was originally isolated from human oral flora by J. M. Jablon *et al.*[10], was supplied by Professor S. Kotani, Department of Microbiology, Osaka University Dental School.

Cultivation and preparation of extracellular water-insoluble glucan. — *S. salivarius* HHT was statically cultivated in a 4-L vessel containing Trypticase soy broth (Baltimore Biological Lab. Inc.) supplemented with 5% of sucrose at pH 7.2 for 20 h at 37°. After cultivation, the culture broth was centrifuged to remove soluble polysaccharide (levan, yield 12.3 g after purification by repeated precipitation with ethanol). The precipitate was washed thoroughly with distilled water, and dissolved in 400 mL of M potassium hydroxide solution containing a small amount of sodium borohydride, by stirring for 3 h at room temperature under a stream of nitrogen. The suspension was centrifuged to remove the cells, and the clear, supernatant solution was made neutral with 6M acetic acid. After 3 h at 4°, the turbid solution was centrifuged to collect the water-insoluble glucan. It was washed thoroughly with distilled water. After one more treatment with alkali, the purified glucan was lyophilized; yield, 450 mg.

Preparation of enzymically synthesized, water-insoluble glucan. — The glucosyltransferase was prepared from 1.7 L of a culture of HHT, grown in Trypticase soy broth in the manner already described. The culture broth was centrifuged to remove the cells, and the supernatant solution was brought to 50% saturation by ammonium sulfate in the presence of 0.05% of egg albumin (Wako Pure Chemical

Industries Ltd., Osaka, Japan) at pH 6.8. The precipitate was collected by centrifugation, dissolved in a minimal volume of 0.05M phosphate buffer (pH 6.8), and dialyzed overnight against the same buffer at 4°. The enzyme activity was calculated on the basis of a determination of reducing sugar produced by incubation of the enzyme with 25mM sucrose in 0.05M phosphate buffer, pH 6.0 (0.2 mL) for 15 min at 37°. The recovery of enzyme activity was 40% from the culture filtrate, and the specific activity was increased 27-fold. The enzyme preparation thus obtained was incubated with a 10% solution of sucrose (400 mL) in 0.05M phosphate buffer (pH 6.8) for 48 h at 37° in the presence of toluene. The turbid mixture was centrifuged, and the insoluble glucan in the precipitate purified as for the extracellular insoluble glucan (yield, 250 mg). From the supernatant of the mixture, a soluble fructan was also obtained (yield, 3.1 g). The low yields of polysaccharides might be attributed to the low activity of the enzyme.

General analytical methods. — The total content of carbohydrate was usually determined colorimetrically by the phenol–sulfuric acid method[11]. D-Glucose was determined by the glucose oxidase method[12]. Reducing sugars were determined by the Nelson–Somogyi method[13]. Protein was measured by the method of Lowry *et al.*[14], using bovine serum albumin as the standard.

Paper chromatography was performed on Toyoroshi No. 50 or Whatman 3MM paper with 6:4:3 (v/v/v) 1-butanol–pyridine–water as the solvent system. Sugars on chromatograms were detected by the alkaline silver nitrate reagent[15].

Gas–liquid chromatography was performed with a Shimadzu Gas Chromatograph GC-6AM. Partially methylated sugar components were separated on a column of 3% ECNSS-M on Gaschrom Q at 180° as their corresponding alditol acetates, or as their methyl glucosides on a column of 15% butanediol succinate polyester on Neosorb N at 175°.

Methylation analysis. — Methylation of the water-insoluble glucan was performed by the method of Hakomori[6]. The glucan sample (~20 mg) under nitrogen was dissolved in dimethyl sulfoxide (2 mL) at room temperature with the aid of ultrasonication. The solution was treated first with methylsulfinyl carbanion (0.5 mL) for 4 h at room temperature, and then with methyl iodide (1.5 mL) for 1.5 h at 20°. The mixture was dialyzed against water and dried *in vacuo*. The methylation procedure was repeated twice, and a portion of the fully methylated glucan was hydrolyzed by heating it first with 90% formic acid for 12 h at 100°, and then with 2M trifluoroacetic acid for 4 h at 100°. The partially methylated sugar components were converted into their alditol acetates, by treatment with sodium borohydride and then with 1:1 pyridine–acetic anhydride. The products were analyzed by g.l.c. Another portion of the methylated glucan was methanolyzed by heating with 3% methanolic hydrogen chloride for 18 h at 100°. The mixture of methyl glucosides was made neutral with silver carbonate and then analyzed by g.l.c.

Periodate oxidation. — Each glucan (100 mg) produced extracellularly and synthesized enzymically was oxidized with 0.08M sodium metaperiodate (100 mL) for 10 days at 25° with stirring in the dark. At suitable time-intervals, the production

of formic acid and the consumption of periodate were determined by titration with 0.01M sodium hydroxide and by the method of Fleury and Lange[16], respectively. When oxidation was complete, the mixture was centrifuged to precipitate the oxidized glucan. The insoluble, oxidized glucan was washed thoroughly with distilled water and reduced by treatment with sodium borohydride for 20 h at room temperature. The insoluble glucan-polyalcohol was collected by centrifugation and a portion of it was hydrolyzed completely by heating it with 2M sulfuric acid for one h at 100°. The hydrolysis products were examined by paper chromatography, g.l.c., and also by l.c. The remainder (50 mg) of the glucan-polyalcohol was hydrolyzed with 0.05M sulfuric acid for 20 h at 25° (mild Smith degradation). The mild hydrolyzate was separated into water-soluble and water-insoluble fractions. The soluble fraction was made neutral, and analyzed by paper chromatography and also by g.l.c., and the insoluble fraction (degraded glucan), which was lyophilized, was subjected to methylation analysis.

ACKNOWLEDGMENTS

We are grateful to Dr. S. Ebisu for his participation in the preliminary work of the research. We thank Professor S. Kotani, Department of Microbiology, Osaka University Dental School, for the supply of the microorganism, and Dr. H. Kumagai, Food Research Institute, Kyoto University, for the ultracentrifugal analysis. This research was supported in part by a Research Grant (No. 156060) from the Ministry of Education, Japan.

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