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Design of quaternary chitosan conjugate having antennary galactose residues as a gene delivery tool

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It is well-known that some kinds of saccharide play the important roles in biological recognition on cellular surface. So, they are expected to be applied for cellular recognition devices. Recently, it was reported that cluster glycosides were effective in the specific interaction between oligosaccharide chains and receptors. Since chitosan is a cationic natural polysaccharide, having formation ability of polyelectrolyte complex with DNA, it is expected to be used as a carrier of DNA in gene delivery systems. So, in order to achieve an efficient gene delivery via receptor-mediated endocytosis, the synthesis of novel polycationic polysaccharide derivative having recognizable branched saccharide residues, *N,N,N*-trimethyl(TM)-chitosan/tetragalactose antenna conjugate (TC-Gal4A20), was carried out. The cellular recognition ability of TC-Gal4A20 conjugate were tested, and then the possibility of its application as a gene delivery tool was investigated. TC-Gal4A20 conjugate showed high affinity to RCA_{120} lectin and its polycation–DNA complex had the ability of specific gene delivery to hepatocyte. © 1997 Elsevier Science Ltd

INTRODUCTION

It is well-known that some kinds of saccharide play the important roles in biological recognition on cellular surface. In particular, the 'cluster effect' referred to by Lee *et al.* (1984), has recently been noted in biological recognition by carbohydrate-receptor bindings in relation to cell–cell interactions (Lee, 1978). They were suggesting that the 'cluster effect', an increase in the binding strength beyond that expected from the increase in galactose concentration, depended upon the maximum spatial inter-galactose distances and the flexibility of the arm connecting galactosyl residues and the branch points (Lee *et al.*, 1984). So, multi-antennary sugar chains are expected to be very effective in the application for cellular recognition devices.

Since many polysaccharides are biodegradable and biocompatible, they are useful as biomedical materials. Chitosan is a cationic, natural polysaccharide which can form polyelectrolyte complex with DNA. So, it is expected to be used as a carrier of DNA in gene delivery systems. However, chitosan itself has no recognizable moiety and low solubility in water except in acidic conditions.

In the previous paper (Murata *et al.*, 1996), we synthesized *N,N,N*-trimethyl(TM)-chitosan/galactose conjugates (TC-Gal5 and TC-Gal20, Fig. 1) and showed the cellular recognition ability and the possibility of those applications for a gene delivery tool. These conjugates had an inclination to increase the cellular recognition ability with the increase of galactose residues in themselves. However, the cellular recognition ability was not very high. Thus, in order to achieve a more efficient gene delivery via receptor-mediated endocytosis, we designed the quaternary chitosan conjugate having antennary galactose residues, TM-chitosan/tetragalactose antenna conjugate (TC-Gal4A20, Fig. 1). In this paper, we are concerned with the cellular recognition ability of TC-Gal5, TC-Gal20 and TC-Gal4A20 conjugates, and the specific gene delivery by these polycation–DNA complex. Moreover, we investigate the cluster effect of tetra-antennary galactose residues and the effect of local galactose concentration in these conjugates.

EXPERIMENTAL

Materials

Chitosan was provided by Kimitsu Chemical Industries, Ltd. Biotin labelled lectin from *Ricinus communis*

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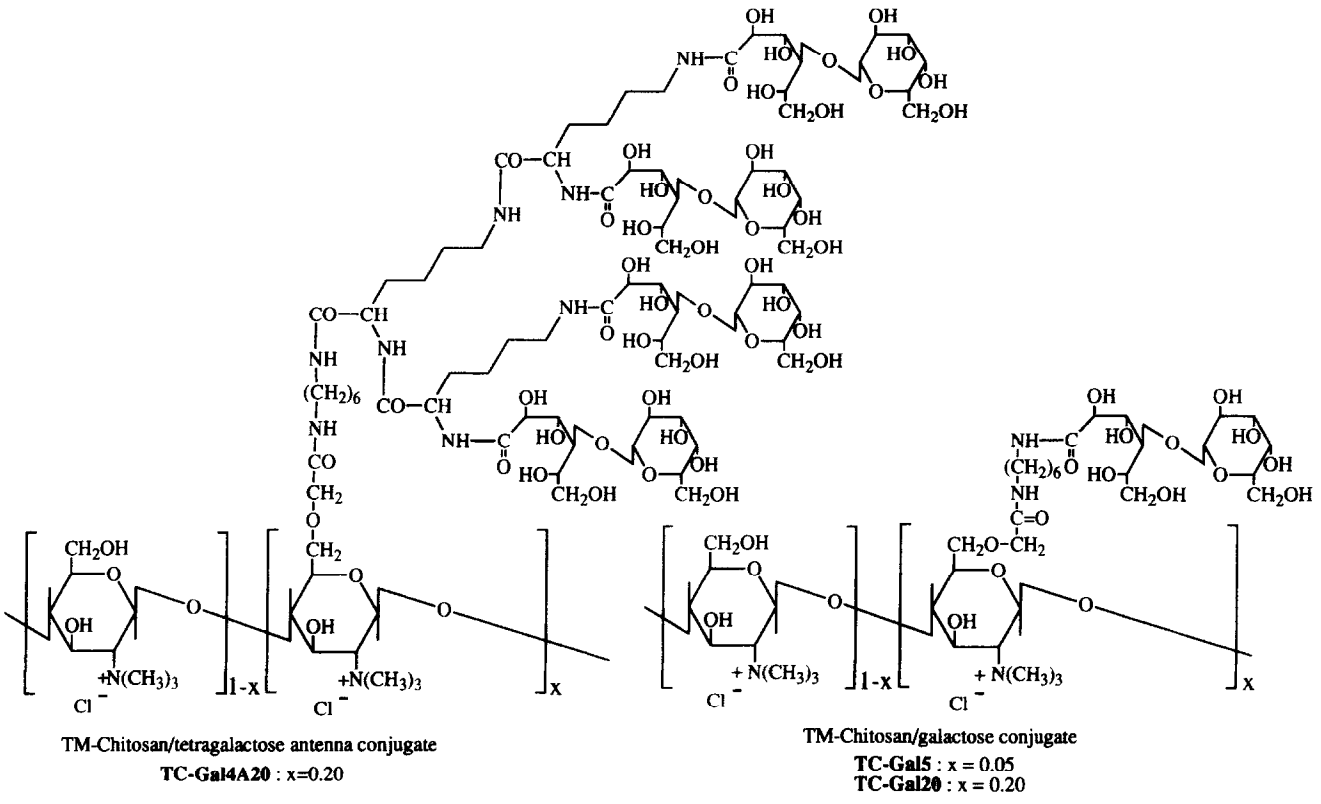


Fig. 1. Molecular structures of TM-chitosan/galactose conjugates and TM-chitosan/tetragalactose antenna conjugate.

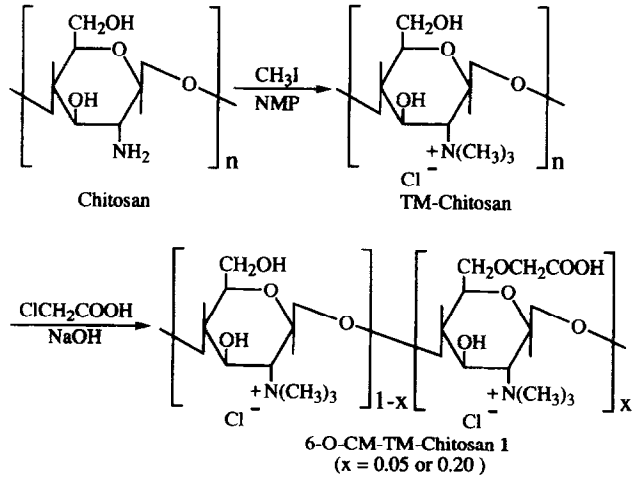
agglutinin-120 (RCA_{120}) was purchased from SIGMA Chemical Company. pSVβGal was purchased from Promega. Organic solvents were purified by usual distillation. Other materials were commercial grade and used without further purification. HepG2 and Hela cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui Seiyaku Co.) containing 10% heat-inactivated fetal calf serum (FCS) (HAZELETON Biologics, Inc.), 4 mM of L-glutamine, 36 mM of sodium bicarbonate and 60 mg/l of kanamycin at 37°C in a humidified atmosphere containing 5% CO_2 in air. The cells using each test were cultured in 96-well flat-bottomed plates (CORNING Laboratory Sciences Company) in 200 μl of culture medium.

Synthesis of TM-chitosan

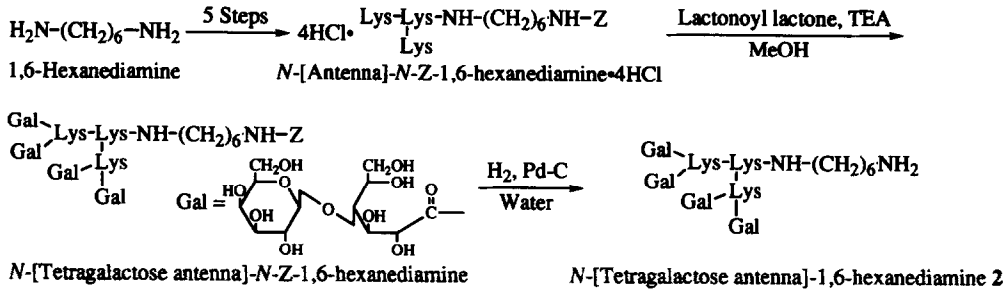
The synthesis of the TM-chitosan unit was performed through two reaction steps, shown in Scheme 1, according to the method described in the previous paper (Murata *et al.*, 1996). TM-chitosan chloride was prepared from chitosan according to the method described in the reference (Domard *et al.*, 1986).

Synthesis of tetragalactose antenna

The synthesis of the tetragalactose antenna residue was performed as shown in Scheme 2. Lactonoyl lactone was prepared from lactose according to the method described in the reference (Kobayashi *et al.*, 1985). *N*-[tetragalactose antenna]-1,6-hexanediamine (**2**) was prepared according to the following method. *N*-[antenna]-*N*-*Z*-1,6-hexanediamine·4HCl (760 mg, 973 μmol), which was synthesized through five reaction steps by the method of peptide synthesis, and lactonoyl lactone (1.60 g, 4.71 mmol) were dissolved in methanol. TEA (600 μl, 4.29 mmol) was added and refluxed for 5 h. This reaction mixture was evaporated and then reprecipitated from water and ethanol. (963 mg, 483 μmol, yield: 49.6%). The *N*-[tetragalactose



Scheme 1. Synthesis route of TM-chitosan.



Scheme 2. Synthesis route of tetragalactose antenna.

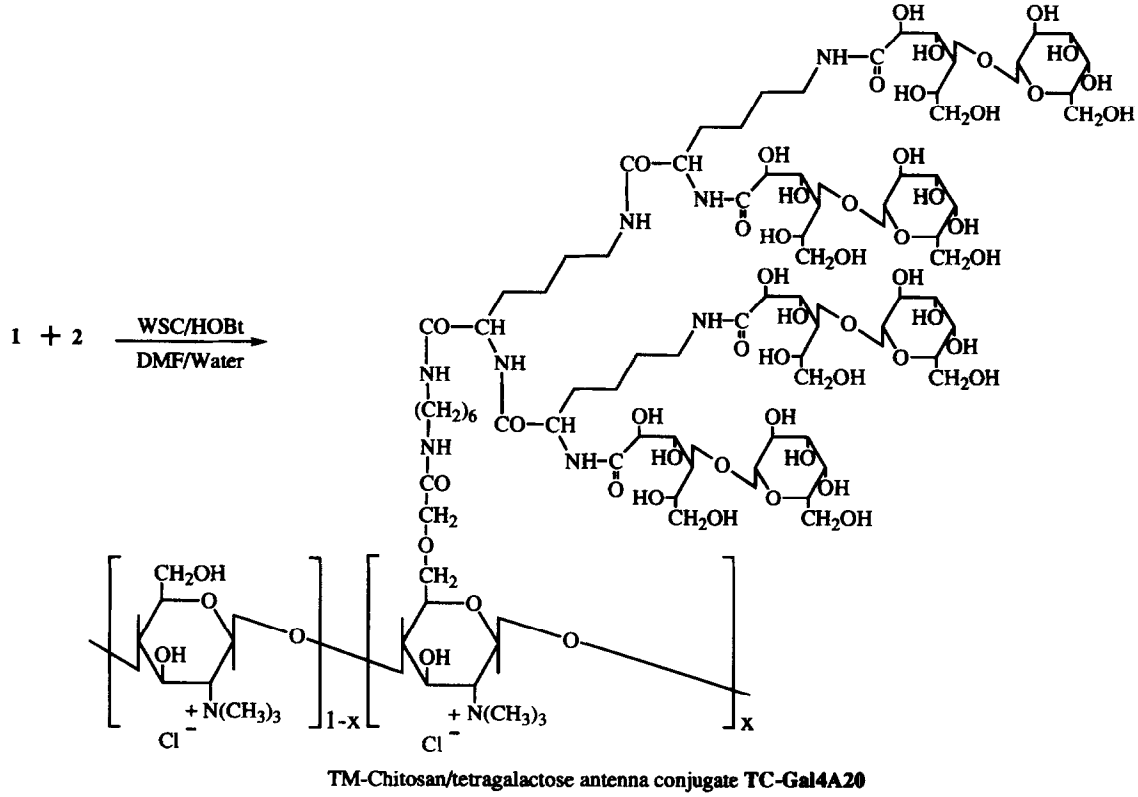
antenna]-*N*-*Z*-1,6-hexanediamine (424 mg, 213 μmol) was dissolved in water and hydrogenolyzed in the presence of palladium black (40 mg) at room temperature for 24 h. After the removal of the catalyst by filtration, the filtrate was evaporated under reduced pressure. *N*-[tetragalactose antenna]-1,6-hexanediamine was isolated by ion-exchange chromatography (SP-Sephadex, conditioning: water, eluent: 0.1 M ammonium bicarbonate), and freeze-dried to be afforded as powder, 378 mg, 202 μmol (yield: 95.0%).

Synthesis of TM-chitosan/tetragalactose antenna conjugate

The synthesis of TM-chitosan/tetragalactose antenna conjugate (the degree of substitution of tetragalactose antenna residue per sugar unit (DGal4A) = 20 mol%/sugar unit: TC-Gal4A20) was performed as shown in Scheme 3. **1** (DCM = 20 mol%/sugar unit, 50.4 mg, 201 μmol/sugar, 40.1 μmol/COOH) was dissolved in DMF/water (1:1 v/v) and added WSC (11.5 mg, 59.9 μmol) and HOBt (6.7 mg, 49.6 μmol) at 0°C for 1 h. Then **2** (90.9 mg, 48.9 μmol) was added and stirred at room temperature over night. After this reaction mixture was evaporated and dissolved in water, it was ultrafiltrated to remove unreacted reagents. The above reaction was repeated until it was lacking in residual carboxyl groups. The objective conjugate was isolated by gel filtration chromatography (HW-40, φ 2×27 cm, eluent: water), and freeze-dried to be afforded as powder, 31.2 mg.

Interaction of polysaccharides with RCA_{120} lectin

The interaction of polysaccharides with RCA_{120} lectin was evaluated using BIA system (BIAcore system,



TM-Chitosan/tetragalactose antenna conjugate TC-Gal4A20

Scheme 3. Synthesis route of TM-chitosan/tetragalactose antenna conjugate.

Pharmacia Biotech) (Shinohara *et al.*, 1995). The biotin labelled RCA_{120} lectin solution was injected onto the streptavidin pre-immobilized surface of a sensor chip, and various concentrations of polysaccharide were injected to measure sensorgrams. The obtained sensorgrams were carried out kinetic analysis of the interaction between RCA_{120} and polysaccharides, the apparent affinity constants of polysaccharides were calculated.

Assay of β-galactosidase activity

The β-galactosidase activity of the conjugates was measured against HepG2 human hepatoma cells *in vitro* according to the method described in the previous paper (Murata *et al.*, 1996). The tumor cell suspension containing 1×10^4 cells in DMEM containing 10% FCS was distributed in a 96-wells microplate and incubated in a humidified atmosphere, containing 5% CO_2 at 37°C for 24 h. After a medium containing 100 μm chloroquine was added and incubated in a humidified atmosphere containing 5% CO_2 at 4°C for 1 h, they were changed to FCS free DMEM containing 5 mM $CaCl_2$ and/or 50 mM lactose for an inhibition test, and added to each sample. After being incubated at 4°C for 1 h, they were changed to FCS free DMEM and incubated for 48 h. After the X-gal solution was added (Takai & Ohmori, 1990), they were incubated for 48 h and measured using a microplate reader at 420 nm.

RESULTS AND DISCUSSION

Synthesis of TM-chitosan/tetragalactose antenna conjugate

In order to expect an efficient binding to a galactose receptor on the surface of hepatocyte, a tetra-antennary ligand having four β-D-galactosyl residues, *N*-[tetragalactose antenna]-1,6-hexanediamine, was designed. Quaternary amine groups in the TM-chitosan/tetragalactose antenna conjugate (TC-Gal4A20) were introduced as binding sites for the polyelectrolyte complex formation with DNA. On the other hand, 6-O-carboxymethyl (CM) groups in the TC-Gal4A20 were introduced as reaction points of the conjugation with tetragalactose antenna residues in order for there to be less steric hindrance to the polyelectrolyte complex formation with DNA. The synthesis of TM-chitosan/tetragalactose antenna conjugate was carried out through reaction steps shown in Schemes 1–3. By the method of peptide synthesis, the *N*-[antenna]-*N*-*Z*-1,6-hexanediamine·4HCl having hetero terminal groups was made from 1,6-hexanediamine. *N*-[tetragalactose antenna]-1,6-hexanediamine having hetero terminal groups, antennary tetragalactose residue and amine, was isolated by ion-exchange chromatography. By the repetition of the reaction of conjugation, the objective TM-chitosan/tetragalactose antenna conjugate, having no residual carboxyl group, was obtained.

Interaction of polysaccharides with RCA_{120} lectin

In order to evaluate the affinity of obtained conjugates to hepatocyte, the interactions between polysaccharides and RCA_{120} lectin were investigated as a simple model experiment. The measurement of interaction between polysaccharides and RCA_{120} lectin was carried out by the use of a biosensor based on surface plasmon resonance (BIAcore system, Pharmacia Biotech) (Shinohara *et al.*, 1995). The results of interaction of polysaccharides with RCA_{120} lectin are shown in Fig. 2. The apparent affinity constant of TM-chitosan was the same level as that of DEAE-dextran. These polysaccharides might be nonspecifically interacted with RCA_{120} lectin, which recognizes β-D-galactose and β-D-*N*-acetylgalactosamine residues. TC-Gal5 and TC-Gal20 were shown the higher apparent affinity constant than TM-chitosan. TC-Gal4A20 was shown the highest apparent affinity constant in these used polysaccharides.

Furthermore, these results were converted into an apparent affinity constant per galactose residue as shown in Fig. 3. TC-Gal20 was shown the lower apparent affinity constant per galactose residue than TC-Gal5. These results might be caused by free galactose residues of TC-Gal20 that were not concerned in the binding with RCA_{120} lectin, those of TC-Gal20 were more than those of TC-Gal5. On the other hand, TC-Gal4A20 was shown as the higher apparent affinity constant per galactose residue than TC-Gal20. These results suggested that the high apparent affinity constant per galactose residue of TC-Gal4A20 was caused by 'cluster effect' referred to by Lee *et al.* (1984). Thus, by the introduction of multi-antennary galactose chains into polysaccharides having no branches, it could be expected that novel polysaccharides, having high affinity to hapatocyte, were able to synthesize.

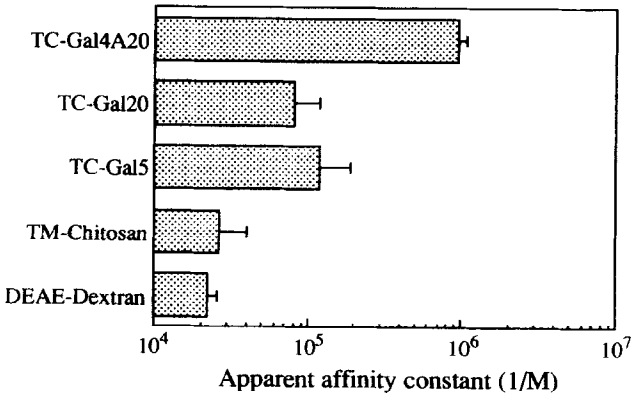


Fig. 2. Apparent affinity constant of polysaccharides against agglutinin RCA_{120}.

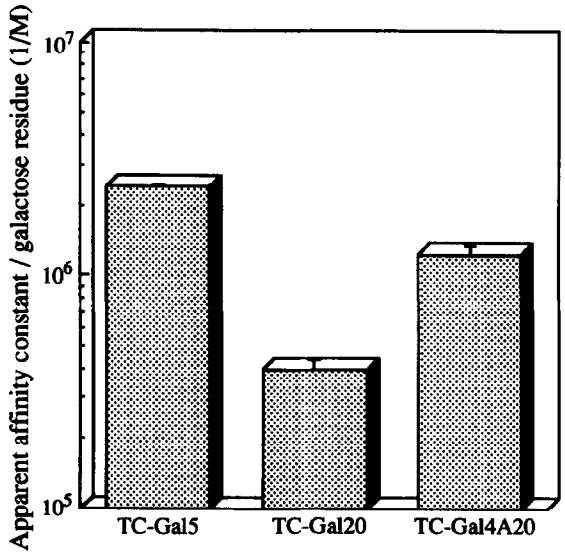


Fig. 3. Apparent affinity constant per galactose residue of polysaccharide against agglutinin RCA_{120}.

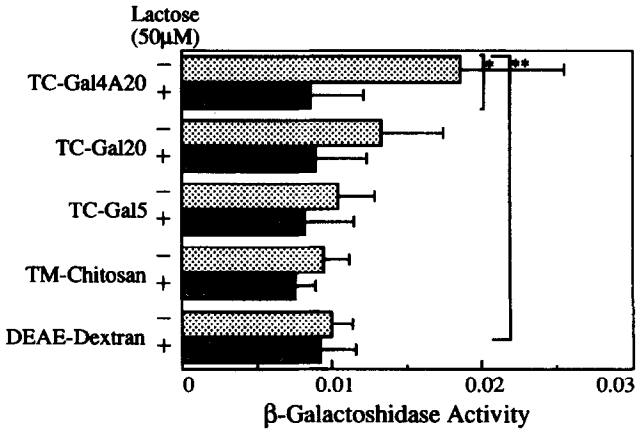


Fig. 4. Effects of inhibitor on expression of β-galactosidase by polycation–DNA complex in HepG2 cells. DNA dose = 0.5 μg/well. Ratio of polycation/DNA = 2 cationic group/1 bp. −: in the absence of inhibitor; +: in the presence of inhibitor; *: $p < 0.0005$; **: $p < 0.01$.

Effects of inhibitor on expression of β-galactosidase activity by polycation–DNA complex in HepG2 cells

In order to investigate the specific delivery of DNA by TC-Gal4A20 conjugate, expression of β-galactosidase activity by polycation–DNA complex in HepG2 cells was carried out. Furthermore, in order to make sure that the specific delivery of DNA by TC-Gal4A20 conjugate was caused by receptor-mediated endocytosis, the inhibition test was performed. The results of the effects of an inhibitor on an expression of β-galactosidase activity by polycation–DNA complex in HepG2 cells are shown in Fig. 4. In the absence of an inhibitor, the β-galactosidase activity of TM-chitosan was the same level as that of DEAE-dextran. TC-Gal5 and TC-Gal20 conjugates were shown to have higher β-galactosidase activity than TM-chitosan and DEAE-dextran. TC-Gal4A20 conjugate was shown to have the highest β-galactosidase activity in these used polysaccharides. With increasing the amount of galactose residues in the conjugate, the β-galactosidase activity of conjugate was increased.

In the presence of an inhibitor, the β-galactosidase activities of DEAE-dextran and TM-chitosan were little affected. On the other hand, those of TC-Gal5, TC-Gal20 and TC-Gal4A20 conjugates were remarkably decreased by the addition of lactose. These results suggested that the increase of β-galactosidase activity of TC-Gal5, TC-Gal20 and TC-Gal4A20 conjugates were caused by the specific internalization via galactose receptor on the cellular surface of HepG2 cells. Therefore, it is very useful for the enhancement of targetting ability that multi-antennary sugar chains are introduced into polysaccharides having no branches.

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