An Acidic Polysaccharide Having Immunological Activities from the Rhizome of *Cnidium officinale*

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An acidic polysaccharide, designated as cnidirhan AG, was isolated from the rhizomes of *Cnidium officinale* MAKINO. It was homogeneous on electrophoresis and gel chromatography, and its molecular mass was estimated to be 5.1×10^4. It showed pronounced reticuloendothelial system–potentiating activity in a carbon clearance test, and had a remarkable effect on both anti-complementary and alkaline phosphatase-inducing activities. It is composed of L-arabinose: D-galactose: D-glucuronic acid in the molar ratio of 2 : 6 : 1, in addition to small amounts of *O*-acetyl groups. Methylation analysis, carbon-13 nuclear magnetic resonance, controlled Smith degradation and limited acid hydrolysis indicated that the core structural features of cnidirhan AG include a backbone chain composed of β-1,3-linked D-galactose residues. Some of the galactose units in the backbone carry β-D-galactosyl side chains at position 6. Both α-L-arabinosyl arabinose side chains and terminal β-D-glucuronic acid residues are linked to the core galactan units.

Keywords *Cnidium officinale*; rhizome; cnidirhan AG; polysaccharide structure; acidic arabinogalactan; immunological activity; reticuloendothelial system; anti-complementary activity; alkaline phosphatase-inducing activity; Smith degradation

The rhizome of *Cnidium officinale* MAKINO (Umbelliferae) is a representative Japanese material of a traditional crude drug used as a remedy for gynopathy and as a tonic under the name of chuanxiong in China (Japanese name, Senkyu). Many volatile alkylphthalide derivatives, pregnenolone and coniferyl ferulate in this crude drug have been reported,[1-3] but no pure polysaccharide with biological activity has so far been obtained. The present paper describes the isolation, structural analysis and three kinds of immunological activities of a novel acidic polysaccharide from the water extract of the rhizome of *C. officinale*.

Material and Methods

Isolation of Polysaccharide The material plant was cultivated in Hokkaido. Sliced dry rhizomes (1000 g) were extracted with hot water (10 l) under stirring for 30 min in a boiling water bath. After centrifugation, the residue was similarly extracted with hot water (5 l). The supernatants were combined and the solution (10 l) was added to 1% sodium sulfate (100 ml); 5% cetyltrimethylammonium bromide (CTAB; 1050 ml) was then added to the solution. After centrifugation, the precipitate obtained was extracted with 0.2 M sodium chloride (2.5 l). After centrifugation, the supernatant was poured into two volumes of ethanol. The precipitate was treated with 80% ethanol, and after centrifugation, the precipitate was dissolved in water, then dialyzed and lyophilized. Yield, 2.4 g. This fraction (2 g) was dissolved in water and applied to a column (4 × 34 cm) of diethylaminoethyl (DEAE)-Sephadex A-25 which had been pretreated as described in a previous report.[4] After elution with water (520 ml), the column was eluted with 0.2 M ammonium carbonate. Fractions of 20 ml were collected and analyzed by the phenol-sulfuric acid method.[5] The eluates obtained from tubes 14 to 36 were combined, dialyzed, concentrated and applied to a column (5 × 78 cm) of Sephadex G-25. The column was eluted with water and fractions of 20 ml were collected. The eluates obtained from tubes 27 to 34 were combined, concentrated and lyophilized. Yield, 194 mg. This fraction (fr. CR-PII, 150 mg) was dissolved in 1/15 M phosphate buffer (pH 7.0) containing 0.15 M NaCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$, and applied to a column (1.5 × 38 cm) of concanavalin A (Con A)-Sepharose (Pharmacia Co.). The column was equilibrated and eluted with the same buffer at 4 °C, and fractions of 10 ml were collected. The eluates obtained from tubes 4 to 9 were combined, dialyzed, concentrated and applied to a column (5 × 82 cm) of Sephadex G-25. The column was eluted with water and fractions of 20 ml were collected. The eluates obtained from tubes 30 to 33 were combined, concentrated and lyophilized. Cnidirhan AG (108 mg) was obtained as a white powder.

Glass-Fiber Paper Electrophoresis This was carried out as described in a previous report[6] on Whatman GF83 glass-fiber paper at 570 V for 1 h with 0.025 M $Na_2B_4O_7 \cdot 10H_2O$–0.1 N NaOH (10 : 1, pH 9.3). Cnidirhan AG gave a single spot at a distance of 122 mm from the origin toward the cathode.

Cellulose Acetate Membrane Electrophoresis This was carried out as described previously[7] for 40 min. The sample gave a single spot at a distance of 98 mm from the origin toward the anode.

Gel Chromatography The sample (3 mg) was dissolved in 0.1 M Tris–HCl buffer (pH 7.0), and applied to a column (2.6 × 85 cm) of Toyopearl HW-55F, pre-equilibrated and developed with the same buffer. Fractions of 5 ml were collected and analyzed by the phenol–sulfuric acid method. Standard pullulans (Shōwa Denkō Co.) having known molecular masses were run on the column to obtain a calibration curve.

Phagocytic Activity This was measured as described in a previous report.[7] The samples and a positive control, zymosan (Tokyo Kasei Co.), were each dissolved and suspended in physiological saline and dosed i.p. (20 mg/kg body weight) to male mice (ICR-SPF) once a day.

Anti-complementary Activity This was measured using normal human serum and IgM-hemolysin-sensitized sheep erythrocytes as described in a previous report.[8] The activity of the sample and a positive control, Plantago-mucilage A from the seed of *Plantago asiatica* L.,[9] were expressed as the percentage inhibition of the residual total hemolytic complement (TCH_{50}) of the control.

Alkaline Phosphatase Assay This was measured using cells from the spleen of ICR-SPF male mice as described in a previous report[10] with some modifications of the amounts of reagents and the reaction time.[11] Lipopolysaccharide from *E. coli* 0111 : B4 (DIFCO Lab.) was used as a positive control. The results were expressed as the arithmetic mean ± S.D. of triplicate cultures.

Qualitative Analysis of Component Sugars Hydrolysis and cellulose thin-layer chromatography (TLC) of component sugars were performed as described in a previous report.[4] The configurations of component neutral sugars were identified by gas chromatography (GC) of trimethylsilylated α-methylbenzylamino-alditol derivatives.[12] GC was carried out on a Shimadzu GC-14A gas chromatograph equipped with a hydrogen flame ionization detector.

Determination of Components Neutral sugars were analyzed by GC after conversion of the hydrolyzate into alditol acetates as described previously.[7] Glucuronic acid was determined by the *m*-hydroxybiphenyl method.[13]

Determination of *O*-Acetyl Groups The sample was hydrolyzed with 0.2 N hydrochloric acid and analyzed by GC using propionic acid as an internal standard as described previously.[14]

Nuclear Magnetic Resonance (NMR) NMR spectrum was recorded on a JEOL JNM A500 FT NMR spectrometer in heavy water containing sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard at 70 °C.

Reduction of Carboxyl Groups The polysaccharide was reduced with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate and sodium borohydride as described in a previous report.[15] The reduction was repeated three times under the same conditions. Yield was 15 mg from 25 mg of cnidirhan AG.

Methylation Analysis Methylation was carried out with powdered sodium hydroxide and methyl iodide in dimethyl sulfoxide as described previously.[16] The yields were 2.8 mg from 2.9 mg of the carboxyl-reduced product and 2.2 mg from 2.1 mg of the Smith degradation product. The products were hydrolyzed with dilute sulfuric acid in acetic acid, then

reduced and acetylated as described in a previous report.[17] The partially methylated alditol acetates obtained were analyzed by gas chromatography–mass spectrometry (GC-MS) using a fused silica capillary column (0.32 mm i.d. × 30 m) of SP-2330 (Supelco Co.) with a programmed temperature increase of 4 °C per min from 160 to 220 °C at a helium flow of 1 ml per min. GC-MS was performed with a JEOL JMS-GX303 mass spectrometer. The relative retention times of the products with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol in GC are listed in Table I.

Controlled Smith Degradation Cnidirhan (70.1 mg) was dissolved in 0.1 N sodium hydroxide (14 ml) and left at room temperature for 10 min. After neutralization with 10 M acetic acid, the solution was adjusted to 17.5 ml with water. Then the oxidation was performed by the addition of the same volume of 0.1 M sodium metaperiodate at 5 °C in the dark. The periodate consumption was measured by a spectrophotometric method.[18] Oxidation was completed after 5 d. The reaction mixture was successively treated with ethylene glycol (0.35 ml) at 5 °C for 1 h and sodium borohydride (370 mg) at 5 °C for 16 h, then adjusted to pH 5.0 by the addition of acetic acid. The solution was concentrated and applied to a column (5 × 87 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 33 to 37 were combined, concentrated and lyophilized. The yield of the product was 67.3 mg.

The product (66.1 mg) was dissolved in 0.5 N sulfuric acid (6.6 ml). After standing at 22 °C for 16 h, the solution was neutralized with barium carbonate. The filtrate was passed through a column (0.7 × 4 cm) of Dowex 50W-X8 (H^+) and eluted with water. The eluate was concentrated and applied to a column (5 × 84 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 31 to 36 were combined, concentrated and lyophilyzed. The yield of the product was 15.8 mg.

Limited Acid Hydrolysis The polysaccharide (10.6 mg) was dissolved in 0.05 M trifluoroacetic acid (1 ml), and the solution was heated at 100 °C for 2 h. The acid was removed by evaporation, then the residue was dissolved in water and applied to a column (2.6 × 91 cm) of Sephadex G-25. The column was eluted with water and fractions of 10 ml were collected. The eluates obtained from tubes 20 to 24 were combined, concentrated and lyophilized. The yield of the de-arabinosylated product was 6.6 mg. Monosaccharides were obtained from the eluates in tubes 38 to 40.

Results

The hot water extract obtained from the rhizome of *Cnidium officinale* was treated with cetyltrimethylammonium bromide in the presence of small amounts of sodium sulfate. The precipitate obtained was extracted with 0.2 M sodium chloride, and the extract was poured into ethanol. Then the precipitate was applied to a column chromatography of DEAE-Sephadex A-25. The eluate with 0.2 M ammonium carbonate was subjected to affinity chromatography on Con A-Sepharose. A pure polysaccharide designated as cnidirhan AG was obtained from the passed-through fraction with a phosphate buffer, followed by dialysis and gel chromatography with Sephadex G-25. The isolation method of the polysaccharide is summarized in Fig. 1.

The polysaccharide gave a single spot on electrophoresis, and gave a single peak on gel chromatography. It has $[\alpha]_D^{23}$ $-13.9°$ (H_2O, $c=0.1$). Gel chromatography gave a value of 5.1×10^4 for the molecular mass.

The effect of cnidirhan AG on the reticuloendothelial

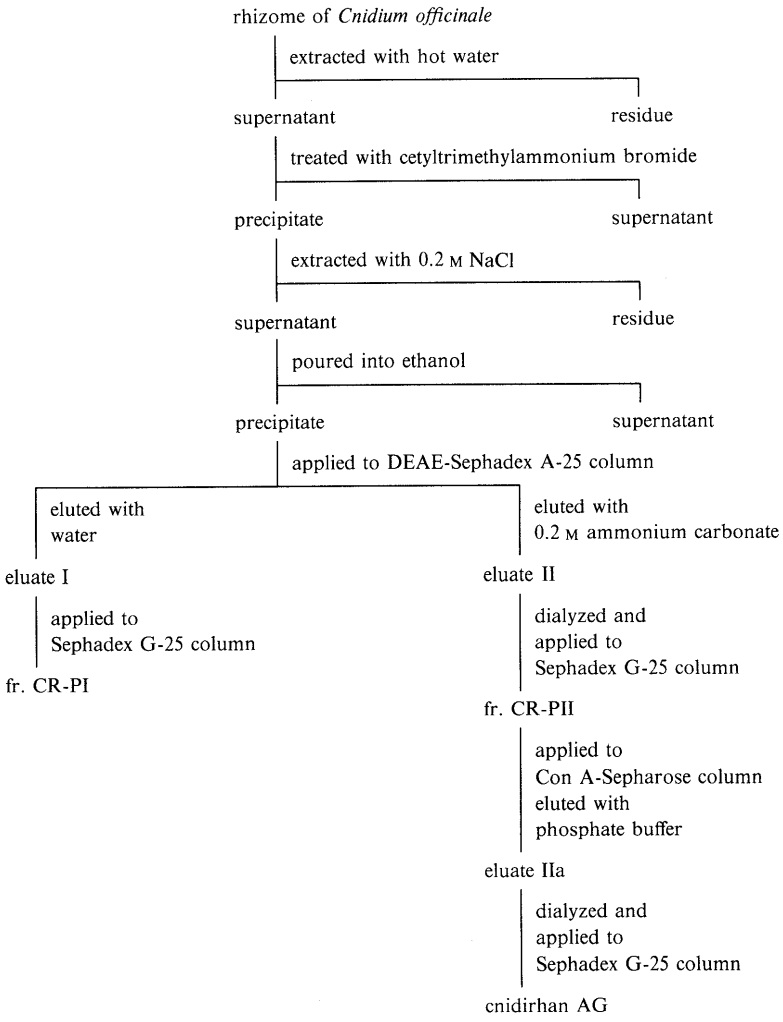

Fig. 1. Isolation of Cnidirhan AG

system (RES) was demonstrated by a modification[7] of the *in vivo* carbon clearance test[19] using zymosan as a positive control. As shown in Fig. 2, the phagocytic index was markedly increased, suggesting activation of the RES by i.p. injection of cnidirhan AG. Thus, the purification of the polysaccharide by affinity chromatography resulted in greater than two-fold rise in the value of the phagocytic index.

The anti-complementary activity of cnidirhan AG is shown in Fig. 3. Cnidirhan AG showed remarkable activity. Further, the measurement of alkaline phosphatase-inducing activity with cnidirhan AG was performed by an *in vitro* murine spleen cell assay, because the activity was induced by stimulating directly with B cell mitogen of indirectly *via* lymphokines with T cell mitogen.[20] As shown in Fig. 4, cnidirhan AG had a significant effect in a dose dependent manner.

Cnidirhan AG is composed of L-arabinose, D-galactose and D-glucuronic acid. It contained no nitrogen. Quantitative analysis showed that it contained 18.6% arabinose, 68.4% galactose and 12.4% glucuronic acid. The molar ratio of these component sugars was 2:6:1.

The carbon-13 NMR (^{13}C-NMR) spectrum of cnidirhan AG showed signals at δ 21.68 and 177.92 ppm, suggesting the presence of *O*-acetyl groups. The presence of acetyl groups (0.53%) was confirmed by GC of the hydrolyzate.

In addition, the ^{13}C-NMR spectrum showed signals due to anomeric carbons at δ 105.30, 106.06, 110.24 and 111.77 ppm. The first and the second signals were assigned to the anomeric carbons of β-D-glucopyranosyluronic acid and β-D-galactopyranose, respectively.[21] The signals at δ 110.24 and 111.77 ppm were assigned to the anomeric carbons of α-L-arabinofuranose.[21]

Methylation analysis of cnidirhan AG gave no good result owing to its poor solubility in dimethyl sulfoxide; no product was able to be identified from the GC-MS of partially methylated alditol acetates. So the carboxyl groups of glucuronic acid residues in the polysaccharide were reduced to give the corresponding neutral sugar residues.[22] The

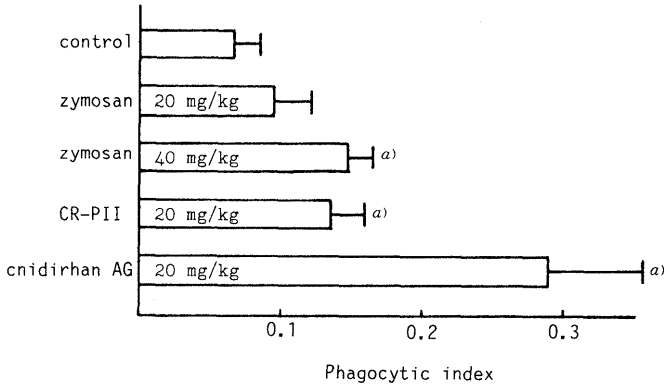

Fig. 2. Effects of Cnidirhan AG and CR-PII on Phagocytosis
Significantly different from the control, *a*) $p<0.001$.

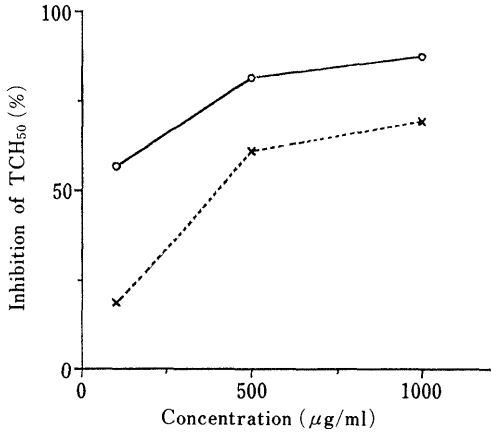

Fig. 3. Anti-complementary Activity of Cnidirhan AG
Cnidirhan AG, —○—; plantago-mucilage A, ---×---.

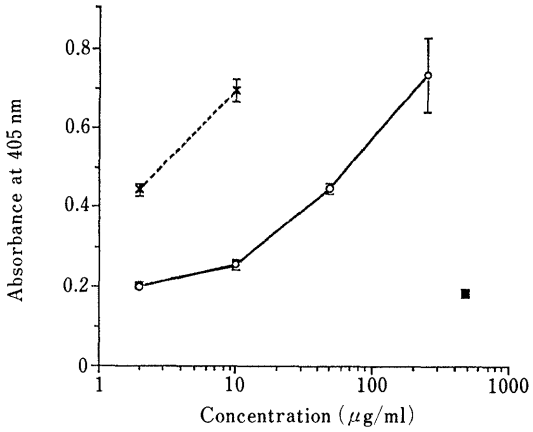

Fig. 4. Mitogenic Activity of Cnidirhan AG Assessed by Alkaline Phosphatase-Inducing Activity
Cnidirhan AG, —○—; lipopolysaccharide, ---×---; nil, ■.

TABLE I. Methylation Analysis of Carboxyl-Reduced Derivative of Cnidirhan AG, Smith Degradation Product and De-arabinosylated Product

Methylated sugars (as alditol acetates)	Relative retention times[a]	Molar ratios			Structural features of original
		Carboxyl-reduced cnidirhan AG	SDP	DeAP	
2,3,5-Me_3-L-arabinose	0.69	7.4	—	0.2	Ara*f* 1→
2,3-Me_2-L-arabinose	1.14	6.6	—	—	→5 Ara*f* 1→
2,3,4,6-Me_4-D-glucose	1.00	7.6	—	—	Glc*p*A 1→
2,3,4,6-Me_4-D-galactose	1.10	1.0	2.2	4.8	Gal*p* 1→
2,4,6-Me_3-D-galactose	1.39	11.0	8.2	15.4	→3 Gal*p* 1→
2,3,4-Me_3-D-galactose	1.62	15.6	1.0	19.3	→6 Gal*p* 1→
2,4-Me_2-D-galactose	2.01	14.5	2.0	10.0	→6 Gal*p* 1→ 3 ↑

a) Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. Abbreviations: Me=methyl; Ara*f*, L-arabinofuranose; Glc*p*A, D-glucopyranosyluronic acid; Gal*p*, D-galactopyranose.

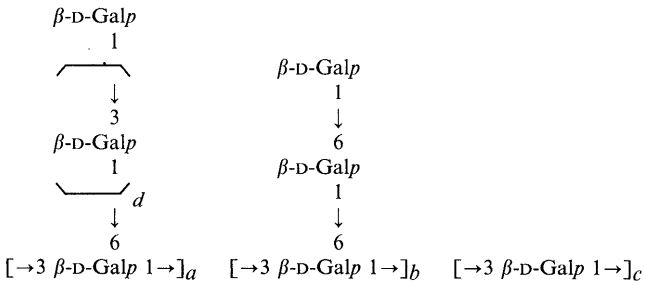

Chart 1. Possible Structural Units of the Smith Degradation Product of Cnidirhan AG

$a:b:(c+d)=1:1:8$

carboxyl-reduced derivative was methylated with solid sodium hydroxide and methyl iodide in dimethyl sulfoxide.[23] The methylated product was hydrolyzed, then converted into partially methylated alditol acetates. Analysis by GC-MS[24] gave the result shown in Table I. The result indicates that D-glucuronic acid residues in the original polysaccharide produced 2,3,4,6-tetra-*O*-methyl D-glucose in the methylation products.

Cnidirhan AG was de-acetylated and subjected to periodate oxidation followed by reduction. The product was treated with dilute sulfuric acid at room temperature overnight.[25] The controlled Smith degradation product (SDP) obtained was composed of D-galactose. SDP was methylated, hydrolyzed, then converted into the partially methylated alditol acetates. Analysis by GC-MS gave the result shown in Table I, thus it indicated the presence of a backbone chain composed of β-1,3-linked D-galactose residues in cnidirhan AG. Some of the galactose residues in the backbone carry galactosyl side chains at position 6. On the basis of the ratio of terminal, intermediate and branching residues, possible structural units of SDP, the core part of cnidirhan AG, are presumed to be as shown in Chart 1.

Limited hydrolysis of cnidirhan AG with very dilute trifluoroacetic acid resulted in the removal of most of the arabinose moiety with some D-galactose residues. The de-arabinosylated degradation product (DeAP) obtained was composed of L-arabinose, D-galactose and D-glucuronic acid in the molar ratio of 1.0:30.5:5.4. The result of methylation analysis of DeAP was also shown in Table I. In this case, the glucuronic acid methyl ether was removed from the products by treatment with anion-exchange resin. Thus, this result revealed an increase in terminal, 1,3-linked and 1,6-linked D-galactose units, and a decrease in 3,6-branched D-galactose units. The accumulated evidence described above indicates that the arabinose units are connected to galactose residues *via* positions 3 and/or 6 in cnidirhan AG. On the other hand, about 90% of arabinose and 4% of galactose units were liberated by the limited hydrolysis. No glucuronic acid was liberated under this condition. In addition, none of the oligosaccharides containing glucuronic acid were found in the hydrolysis products. These results revealed that the terminal glucuronic acid units are directly connected to the core galactose units in the polysaccharide.

Discussion

During our studies on the immunologically active polysaccharides in crude drugs obtained from various plant sources, 20 substances have so far been isolated and characterized as RES-activating polysaccharides. Among them, acidic arabino-3,6-galactan is a representative group. We have already obtained saposhnikovan A,[7] MVS-IIIA, -IVA and -VI,[26-28] ukonans A, B and C,[29-31] glycyrrhizans UA, UB and GA,[21,32] eucomman A,[33] and AMon-S[34] as examples of active polysaccharides belonging to this group.

Most of them have D-galacturonic acid as the sole component hexuronic acid. Glycyrrhizan GA and AMon-S, however, possess both D-galacturonic acid and D-glucuronic acid as their components. In each of the immunologically active acidic arabinogalactans obtained by us, D-galacturonic acid is generally present as an intermediate unit having α-1,4-linkage. Some of the polysaccharides have additional 2,4- or 3,4-branched galacturonic acid residues. On the other hand, D-glucuronic acid forms β-linked terminal units in both glycyrrhizan GA and AMon-S.

Cnidirhan AG is a typical α-1,5-linked L-arabino-β-3,6-branched D-galactan. The ratio of arabinogalactan moieties in the polysaccharide amounts to nearly 90%. In addition, this substance can be classified under a novel type of acidic arabino-3,6-galactan having high immunological activity. Its component hexuronic acid is not D-galacturonic acid, but D-glucuronic acid. Recently, we elucidated the core galactan structures of ukonan A,[35] glycyrrhizan UA[36] and MVS-VI[37] having backbone chains composed of β-1,3-linked D-galactose residues in which the majority of them carry galactosyl side chains at position 6. Thus, the core galactan structure of cnidirhan AG is similar to those of ukonan A, glycyrrhizan UA and MVS-VI. Further studies of relationship between the structure of cnidirhan AG and its activities are in progress.

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