International Journal of Medical, Medicine and Health Sciences
ISSN: 2517-9969
Vol:10, No:2, 2016

An Antibacterial Dental Restorative Containing 3,4-Dichlorocrotonolactone: Synthesis, Formulation and Evaluation

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***Abstract*—**The objective of this study was to synthesize and characterize 5-acryloyloxy-3,4-dichlorocrotonolactone (a furanone derivative), use this derivative to modify a dental restorative, and study the effect of the derivative on the antibacterial activity and compressive strength of the formed restorative. In this study, a furanone derivative was synthesized, characterized, and used to formulate a dental restorative. Compressive strength (CS) and *S. mutans* viability were used to evaluate the mechanical strength and antibacterial activity of the formed restorative. The fabricated restorative specimens were photocured and conditioned in distilled water at 37°C for 24 h, followed by direct testing for CS or/and incubating with *S. mutans* for 48 h for antibacterial testing. The results show that the modified dental restorative showed a significant antibacterial activity without substantially decreasing the mechanical strengths. With addition of the antibacterial derivative up to 30%, the restorative kept its original CS nearly unchanged but showed a significant antibacterial activity with 68% reduction in the *S. mutans* viability. Furthermore, the antibacterial function of the modified restorative was not affected by human saliva. The aging study also indicates that the modified restorative may have a long-lasting antibacterial function. It is concluded that this experimental antibacterial restorative may potentially be developed into a clinically attractive dental filling restorative due to its high mechanical strength and antibacterial function.

***Keywords*—**Antibacterial, dental filling restorative, compressive strength, *S. mutans* viability.

I. Introduction

LONG-LASTING restoratives and restoration are clinically attractive because they can reduce patients' pain and expense as well as the number of their visits to dental offices [1]-[4]. In dentistry, both restorative materials and oral bacteria are believed to be responsible for the restoration failure [2]. Secondary caries is found to be the main reason to the restoration failure of dental restoratives such as resin composites [1]-[4]. Secondary caries that often occurs at the interface between the restoration and the cavity preparation is primarily caused by demineralization of tooth structure due to invasion of plaque bacteria (acid-producing bacteria) such as *Streptococcus mutans* (*S. mutans*) and lactobacilli in the presence of fermentable carbohydrates [4]. To make long-lasting restorations, the materials should be made antibacterial. Although numerous efforts have been made on improving antibacterial activities of dental restoratives, most of them have been focused on release or slow-release of various incorporated low molecular weight antibacterial agents such as antibiotics, zinc ions, silver ions, iodine and chlorhexidine [5]-[9]. Yet release or slow-release can lead or has led to a reduction of mechanical properties of the restoratives over time, short-term effectiveness, and possible toxicity to surrounding tissues if the dose or release is not properly controlled [5]-[9]. Materials containing quaternary ammonium salt (QAS) or phosphonium salt groups have been studied extensively as an important antimicrobial material and used for a variety of applications due to their potent antimicrobial activities [10]-[14]. These materials are found to be capable of killing bacteria that are resistant to other types of cationic antibacterials [15]. The examples of the QAS-containing materials as antibacterials for dental restoratives include incorporation of a methacryloyloxydodecyl pyridinium bromide as an antibacterial monomer into resin composites [12], use of methacryloxylethyl cetyl ammonium chloride as a component for antibacterial bonding agents [16], [17], and incorporation of quaternary ammonium polyethylenimine nanoparticles into resin composites [18], [19]. All these studies found that the QAS-containing materials did exhibit significant antibacterial activities. However, our recent study found that incorporation of QAS into dental resin composites can significantly decrease mechanical strengths due to its strong hydrophilic characteristics, if the amount added is beyond a certain limit [20]. In addition, it has been reported that human saliva can significantly reduce the antibacterial activity of the QAS-containing restoratives, probably due to electrostatic interactions between QAS and proteins in saliva [21], [22]. Recently furanone derivatives have been found to have strong antitumor [23] and antibacterial functions [24]. In the study, we would like to explore them in dental applications.

The objective of this study was to synthesize and characterize a furanone derivative, use this derivative to modify the dental filling restorative, and study the effect of the derivative on the antibacterial activity and compressive strength of the formed restorative.

II. Experimental

A. Materials

Bisphenol A glycerolate dimethacrylate (BisGMA), Bisphenol A ethoxylate dimethacrylate (BisEMA), urethane dimethacrylate (UDMA), dl-camphoroquinone, 2-(dimethylamino)ethyl methacrylate, toluene, acryloyl chloride (AC), 2,3-dichloromalealdehydic acid (DCA),

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2,3-dibromomalealdehydic acid (DBA), ethyl acetate and sodium bicarbonate were used as received from Sigma-Aldrich Co. (Milwaukee, WI) without further purifications. The untreated glass fillers from Herculite XRV (0.7 microns) were used as received from Sybron Dental Specialties (Newport Beach, CA).

B. Synthesis and Characterization

5-acryloyloxy-3,4-dichlorocrotonolactone (ADCC) - a new monomer was prepared from the reaction of DCA with AC in the presence of toluene at 90-100°C for 3-4 h. After toluene was removed, the residue was washed with sodium bicarbonate and distilled water, followed by extracting with ethyl acetate. ADCC was purified by completely removing ethyl acetate. The monomer, 5-acryloyloxy-3,4-dibromocrotonolactone (ADBC), was synthesized similarly. The synthesis scheme is shown in Fig. 1. The chemical structure of the synthesized ADCC and starting chemicals was characterized by Fourier Transform-Infrared (FT-IR) spectroscopy and nuclear magnetic resonance (NMR) spectroscopy. The proton NMR (^{1}HNMR) spectra were obtained on a 500 MHz Bruker NMR spectrometer (Bruker Avance II, Bruker BioSpin Corporation, Billerica, MA) using deuterated dimethyl sulfoxide and chloroform as solvents and FT-IR spectra were obtained on a FT-IR spectrometer (Mattson Research Series FT/IR 1000, Madison, WI).

C. Fabrication of Specimens

The antibacterial dental restorative was prepared as described previously [20]. Briefly, the restorative was formulated with a two-component system (liquid and powder). The liquid was formulated with dl-camphoroquinone (photo-initiator, 1% by weight), 2-(dimethylamino)ethyl methacrylate (activator, 2%), ADCC, BisGMA, UDMA and BisEMA, where ADCC/a mixture (BisGMA/UDMA/BisEMA = 1:1:1, by weight) = 0, 5, 10, 20, 30, 40, 50 and 70% (by weight). The schematic structures of BisGMA, BisEMA and UDMA are shown in Fig. 1. The untreated glass powders (Herculite XRV, 0.7 microns) were used as fillers and treated with γ-(trimethoxysilyl)propyl methacrylate as described elsewhere [20]. A filler level at 75% (by weight) was used throughout the study. After mixing, the restorative was filled into glass tubing to form a cylindrical specimen (4 mm in diameter by 8 mm in length) for CS and a disk-shape specimen (4 mm in diameter by 2 mm in depth) for antibacterial tests. Specimens were exposed to blue light (LED, 30W, EXAKT 520 Blue Light Polymerization Unit, EXACT Technologies, Oklahoma City, OK, USA) for 2 min [20].

D. Strength Measurement

The CS test was performed on a screw-driven mechanical tester (QTest QT/10, MTS Systems Corporation, Eden Prairie, MN, USA) with a crosshead speed of 1 mm/min [20]. The sample sizes were n = 6-8 for each formulation. CS was calculated using an equation of $CS = P/\pi r^2$, where P = the load at fracture and r = the radius of the cylinder.

E. MIC Test

The minimal inhibitory concentration (MIC) of the synthesized antibacterial monomers was determined following the published protocol [25]. Briefly, colonies of *S. mutans* (UA159) were suspended in 5 ml of Tryptic Soy Broth (TSB) prior to MIC testing. Two-fold serial dilutions of the synthesized monomer were prepared in TSB, followed by placing in 96-well flat-bottom microtiter plates with a volume of 250 µl per well. The final concentration of the monomer ranged from 1.563 to 75µg/ml. The microtiter plate was then inoculated with *S. mutans* suspension (cell concentration = 5 x 10^5 CFU/ml) and incubated at 37°C for 48 h prior to MIC testing. The absorbance was measured at 595 nm via a microplate reader (SpectraMax 190, Molecular Devices, CA) to assess the cell growth. Chlorhexidine was used as control [25]. Triple replica was used to obtain a mean value for each material. The MIC test against lactobacilli was determined similarly.

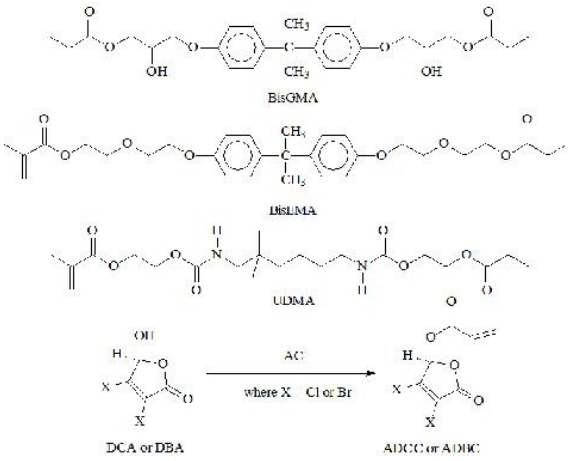

Fig. 1 Schematic diagram for the structures of the oligomers used in the study and synthesis of ADCC or ADBC from the reaction of DCA or DBA with AC

F. Antibacterial Test

The antibacterial test was conducted following the published procedures [20]. *S. mutans* was used to evaluate the antibacterial activity of the studied restoratives. Briefly, colonies of *S. mutans* were suspended in 5 ml of tryptic soy broth (TSB), supplemented with 1% sucrose, to make a suspension with 10^8 CFU/ml of *S. mutans*, after 24 h incubation. Specimens pretreated with ethanol (10 s) were incubated with *S. mutans* in TSB at 37°C for 48 h under 5% CO_2. After equal volumes of the red and the green dyes (LIVE/DEAD BacLight bacterial viability kit L7007, Molecular Probes, Inc., Eugene, OR, USA) were combined in a microfuge tube and mixed thoroughly for 1 min, 3 µl of the dye mixture was added to 1 ml of the bacteria suspension, mixed by vortexing for 10 s, sonicating for 10 s as well as vortexing for another 10 s, and kept in dark for about 15 min, prior to analysis. Then 20 µl of the stained bacterial suspension was analyzed using a fluorescent microscope (Nikon Microphot-FXA, Melville, NY, USA). Triple replica was used to obtain a

mean value for each material. The antibacterial test against lactobacilli was determined similarly. For evaluation of the saliva effect, human saliva (obtained from a healthy volunteer) was centrifuged for 15 min at 12,000g to remove debris [21]. After the supernatant was filtered with a 0.45-μm sterile filter, the filtrate was stored in a freezer (-20°C) prior to testing. The sterilized restorative specimen was incubated in a small tube containing 1 ml of saliva at 37°C for 2 h [21], followed by placing in 5 ml TSB supplemented with 1% sucrose. The rest of the procedures were the same as above.

G. Aging of Specimens

The specimens for both CS and antibacterial activity aging tests were conditioned in distilled water at 37°C for 1 day, 3 days and 7 days, followed by direct testing for CS (see subsection *D*) and incubating with *S. mutans* for 48 h for antibacterial testing (see subsection *F*).

H. Statistical Analysis

One-way analysis of variance (ANOVA) with the post hoc Tukey-Kramer multiple-range test was used to determine significant differences of both CS and antibacterial tests among the materials in each group. A level of $\alpha = 0.05$ was used for statistical significance.

III. Results and Discussion

A. Characterization

Furanone-containing materials are reported to have a broad range of biological and physiological properties including antitumor, antibiotic, haemorrhagic and insecticidal activity [23]-[26]. The biological mechanism of these derivatives is still under investigation [24]. To explore the application of these compounds in dental research, we synthesized a photocurable furanone derivative and applied it to dental filling restoratives.

TABLE I
Characteristic Peaks from FTIR and ^{1}HNMR Spectra

Material	The peaks from FT-IR & chemical shifts from ^{1}HNMR
	The characteristic peaks (cm^{-1}) from FT-IR
DCA	3362 (OH), 1766 (C=O), 1644 (C=C), 1332, 1237 and 949 (C-O-C on pseudo ester), 1451, 1026 and 778 (pseudo –OH), 1279, 1118, 889 and 602 (C-O on pseudo C-OH), 746 (C-Cl)
AC	1758 (C=O on carbonyl group), 1610 (C=C), 1395 and 1145 (C-H on –C=C-), 1284, 1074, 935 and 606 (C-O on carbonyl group), 971 and 755 (C-H on –C=C), 705 (C-Cl)
ADCC	1807 and 1764 (C=O on carbonyl groups), 1639 (C=C on acrylate and internal C=C), 1500 (C-O-C), 1407 and 1137 (C-H on C=C), 1330 and 1232 (C-O-C on pseudo ester), 1295, 1068, 934 and 608 (C-O on carbonyl group), 985 (C-H on –C=C), 889 (C-O on newly formed ester), 804 and 670 (C-H on newly formed C=C), 745 (C-Cl on Cl-C=C)
	The characteristic chemical shifts (ppm) from ^{1}HNMR
DCA	DCA: 6.25 (-CH) and 3.45 (-OH)
AC	6.21, 6.05 and 5.82 (H_2C=CH-)
ADCC	7.20 (-CH), 6.55, 6.30 and 6.15 (H_2C=CH-)

Table I shows the FT-IR spectra for DCA, AC and ADCC. The disappearance of the peak at 3362 for pseudo hydroxyl group on DCA and appearance of the new peaks at 1807, 1764, 1500, 804 and 670 for both carbonyl and C=C groups on acrylate confirmed the formation of ADCC. Table I also shows the ^{1}HNMR spectra for DCA, AC and ADCC. The chemical shift at 2.50 shown in all the spectra was for solvent d-DMSO. The disappearance of the chemical shift at 3.45 (-OH) and all the chemical shifts towards a high field confirmed the formation of ADCC.

B. Evaluation

Fig. 2 shows the effect of the chlorine-containing antibacterial derivative or ADCC content on CS and *S. mutans* viability of the experimental restorative. For CS, the ADCC addition did not change the CS of the restorative until reaching 40%. From 40% to 70%, CS decreased 11-27% of its original value. For the *S. mutans* viability, increasing the ADCC content significantly decreased the *S. mutans* viability. The mean viability values were from 82 to 1% with 5 to 70% ADCC addition, where all the values were significantly different from each other ($p < 0.05$). The result indicates that this new furanone derivative has potent antibacterial activity. In addition, if we incorporate it within 30-40%, the CS of the restorative can be kept nearly unchanged. The result suggests that we may incorporate the new furanone derivative up to 30% to maximize the antibacterial activity without reducing mechanical strengths, which is clinically favorable.

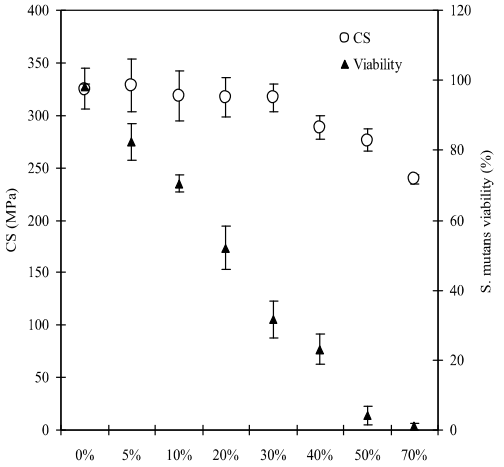

Fig. 2 Effect of the ADCC content on CS and *S. mutans* viability of the experimental restoratives: ADCC content (%, by weight) = ADCC/(ADCC/BisGMA/UDMA/BisEMA), where BisGMA/UDMA/BisEMA = 1:1:1; The filler/resin ratio = 3.0 or 75% (by weight). For CS, specimens were directly used for the testing. For the *S. mutans* viability, specimens were incubated with *S. mutans* for 48 h before antibacterial testing

Table II shows the MIC values of ADCC, bromine-containing derivative (ADBC) and chlorhexidine against *S. mutans* and lactobacillus as well as bacterial viability of these two oral bacteria after culturing with ADCC and ADBC-modified restoratives. The MIC values against *S. mutans* and lactobacillus were 6.25 and 18.7, 9.36 and 37.4, and 1.56 and 6.25, respectively, for ADCC, ADBC and chlorhexidine. The viability values of both oral bacteria after culturing with ADCC and ADBC ranged from 31.6 to 36.4, among which there were no significant differences, although lactobacillus showed higher values than *S. mutans* and ADBC showed higher values than ADCC. The results from MIC and viability data suggest that both ADCC and ADBC are good antibacterial derivatives and capable of killing *S. mutans* and lactobacillus, even though the former seems better than the latter.

TABLE II
MIC OF THE MATERIALS AND BACTERIAL VIABILITY

Compound[1]	*S. mutans*	Lactobacillus
	MIC value[2] (μg/ml)	
ADCC	6.25	18.7
ADBC	9.36	37.4
Chlorhexidine	1.56	6.25
	Viability[3] (%)	
ADCC	31.6 (9.0)	35.3 (4.8)
ADBC	34.2 (2.4)	36.4 (3.7)

[1]ADCC and ADBC are the abbreviations of antibacterial furanone derivatives, which can be found under Materials and Methods; [2]MIC values were measured as shown under Materials and Methods. [3]The restorative formulation was the same as those described in Fig. 4, except for ADCC or ADBC content = 30%; [3]Entries are mean values with standard deviations in parentheses. Specimens were conditioned in distilled water at 37 °C for 24 h, followed by incubating with *S. mutans* or lactobacillus for 48 h for antibacterial testing.

Table III shows the effect of both ADCC and ADBC on CS and *S. mutans* viability of the restoratives. Like those ADCC in Fig. 2, increasing the loading of ADBC decreased the CS values of the restorative and *S. mutans* viability. By comparison, it is clear that at the same loading the ADBC-modified restoratives showed statistically lower CS values than the ADCC-restoratives, although both were not statistically significant different from each other in antibacterial activity. Due to the smaller size of chlorine, we hypothesized that the ADCC-modified restoratives might favor the mechanical strength as compared to the ADBC-restoratives, although we did not know if their antibacterial activity would be different. The result in Table III shows that the ADCC-modified restoratives were statistically significantly higher in CS than the ADBC-modified restoratives, indicating that our hypothesis was correct, i.e., smaller chlorine favors CS. However, no significant differences in antibacterial activity were found between two modified restoratives. The result suggests that the ADCC-modified restoratives might be a better choice for restorative formulation on behalf of CS and antibacterial tests.

TABLE III
EFFECT OF ADCC AND ADBC ON CS AND *S. MUTANS* VIABILITY OF THE RESIN COMPISITES[1]

Material	CS (MPa)		*S. mutans* viability [%]	
	ADCC	ADBC	ADCC	ADBC
0	325.1 (19)^{a,2}	325.1 (19)	98.3 (0.8)	98.3 (0.8)
5%	328.6 (21)^{a}	309.4 (11)^{b}	82.4 (5.2)^{B}	86.5 (3.4)^{B}
10%	318.5 (22)^{a}	291.2 (9.7)^{b,c}	70.5 (2.4)^{C}	74.4 (5.4)^{C}
30%	317.0 (13)^{a}	285.4 (8.5)^{c}	31.6 (9.0)^{E}	32.1 (2.3)^{E}

[1]The formulations were the same as those described in Fig. 2, except that ADCC contains chlorine and ADBC contains bromine; [2]Entries are mean values with standard deviations in parentheses and the mean values with the same superscript letter were not significantly different ($p > 0.05$). Specimens were conditioned in distilled water at 37°C for 24 h, followed by direct testing for CS or/and incubating with *S. mutans* for 48 h before antibacterial testing.

Fig. 3 shows the effect of human saliva on the *S. mutans* viability after culturing with the modified restorative. No statistically significant differences in the *S. mutans* viability were found between the restoratives with and without human saliva treatment. It was reported that saliva can significantly reduce the antibacterial activity of the QAS or PQAS-containing materials based on the mechanism of contact inhibition [21], [22]. Due to saliva coating or protein film formation on the antibacterial surface of the material, the antibacterial capability became less effective [21], [22]. The reduction was attributed to the interaction between positive charges on QAS or PQAS and amphiphilic protein macromolecules in saliva. Unlike QAS or PQAS, ADCC does not carry any charges. That may be why the ADCC-modified restorative did not show any reduction in antibacterial activity after treating with saliva.

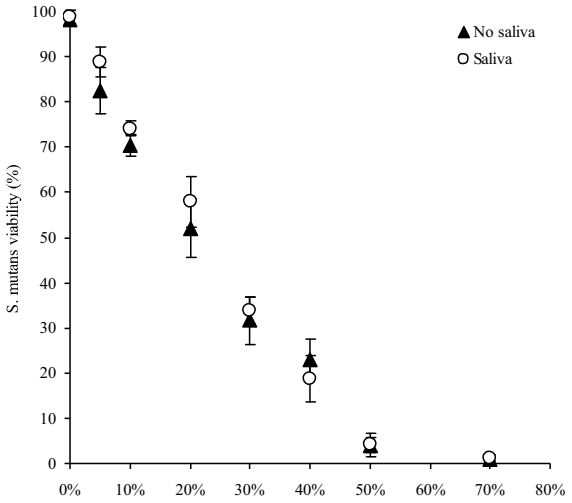

Fig. 3 Effect of human saliva on the *S. mutans* viability after culturing with the restoratives: The formulations were the same as those described in Fig. 2. Specimens were soaked in human saliva at 37 °C for 2 h, followed by incubating with *S. mutans* for 48 h before antibacterial testing

Fig. 4 shows the effect of the modified restorative aging in water on CS and *S. mutans* viability. After 7-day aging in water,

all the restorative specimens with ADCC addition showed no statistically significant differences in either CS or *S. mutans* viability from one another ($p > 0.05$). It is known that dental resin restoratives show a certain degree of degradation due to water sorption caused by two hydroxyl groups pendent on BisGMA and three -CH_2CH_2O- units on triethylene glycol dimethacrylate [27]. The absorbed water can hydrolyze the silane bond that is used to couple resin with fillers, de-bond the resin-filler interface, and thus reduce the mechanical strengths with time [27]. Our previous study found that using QAS to modify the restorative could significantly decrease CS probably due to strong hydrophilic nature of the QAS incorporated [20]. The ionic charges on QAS or PQAS can accelerate the interfacial de-bonding [20]. However, the furanone derivative-modified restorative did not show any statistically noticeable change in CS, indicating that the newly synthesized antibacterial furanone derivative seems more suitable to formulating resin restoratives than antibacterial QAS [20], probably due to the hydrophobicity of the former. The result might also imply that the ADCC-modified resin restorative can have a long-lasting antibacterial function, because otherwise the restorative would lose its CS if ADCC was leachable.

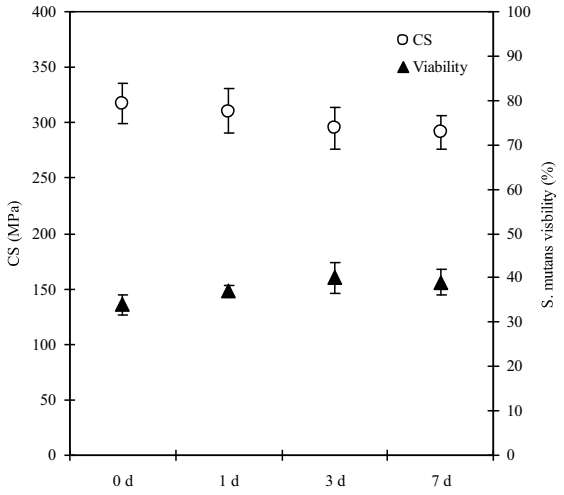

Fig. 4 Effect of aging on CS and the *S. mutans* viability of the experimental restorative: The formulation was the same as those described in Fig. 2, except for ADCC content = 30%. Specimens were conditioned in distilled water at 37°C for 1, 3 and 7 days, followed by direct testing for CS or/and incubating with *S. mutans* for 48 h before antibacterial testing

IV. Conclusions

In conclusion, we have developed a novel antibacterial dental restorative with comparable mechanical strength and long-lasting antibacterial function. Within the limitations of this study, the developed experimental restorative may be clinically attractive because all dental restoratives need to be long lasting. Our future studies will include evaluation of other mechanical and physical properties and biocompatibility of the experimental restorative.

Acknowledgments

This work was partially sponsored by NIH grant DE020614. Dr. Gregory at IU School of Dentistry is appreciated for his support in antibacterial testing.

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