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Evaluation of the Antibacterial Properties of Depsidones Against *Pseudomonas aeruginosa*

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Abstract

The world is facing an antibiotic resistance crisis that will result in millions of deaths across the world per year if current trends continue. In the United States alone, about 2.8 million people contract an antibiotic resistant infection per year. This current health emergency has established that there is a need for the discovery and development of new effective antibiotics to treat multidrug resistant pathogens. This work aimed to determine whether the analogs of depsidone, a natural product isolated from lichen, could be used as a potential treatment against Gram-positive and Gram-negative antibiotic resistant bacteria. Results showed that the core structure of the depsidone, 6,7,6-fused ring system, proved to be essential to the potency of the antibacterial activity. In this study, the methylated depsidone had 5% cell death against *Pseudomonas aeruginosa*. Structure activity relationship studies against various resistant bacteria, similar to *Staphylococcus aureus* strains, revealed that a suitable hydrophobic acyl tail in the phloroglucinol scaffold is a prerequisite for antibacterial activity.

1. Introduction

Antimicrobial resistance (AMR) is a growing global health threat. The World Health Organization has declared AMR to be the fifth leading global health threat facing humanity in 2020.[1] Ironically, the increase in bacterial resistance is caused by individual overuse or misuse of antibiotics and overuse of antibiotics in agriculture. When an individual takes an antibiotic incorrectly by not completing the drug course, some of the harmful bacteria in the body die, but some of the resistant bacteria, which have a natural genetic mutation that makes them less susceptible to the antibiotic, survive and multiply making it difficult for current antibiotics to kill or neutralize the resistant bacteria.

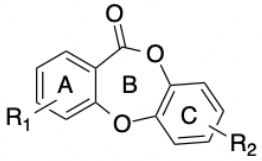
AMR can occur naturally over time, through genetic changes. Since most of the antibiotics that are being used today were identified and used to treat diseases during the 1940's- 1960's the resistance was inevitable. Charles Darwin's theory of natural selection proclaims that the heritable traits of a species that favors survival and reproduction are passed on from generation to generation resulting in evolution in a species over time. Hence, the bacteria from the 1940's-1960's have undergone random mutations in the past 81 years that have allowed them to survive the antibiotic treatments that once killed them, leaving the resistant bacteria untreatable. The current issue is that people from all over the world are dying at an alarming rate because of the shortage of effective antibiotics. In fact, it's projected that over 10 million deaths will occur per year worldwide by 2050.[1] This is why the World Health Organization (WHO) has declared there is a greater need for discovering new antibiotics and antimicrobial medicine against human pathogenic bacteria.[2] For instance, the rate of resistance within ciprofloxacin from 1998 to 2002 increased steadily from 46.6% to 59.4%.[3]

More than 75% of antibiotics were originally derived from natural products.[4] One of the major sources of these natural products are microorganisms, which produce antibiotics at low concentrations, to inhibit other competing microorganisms in nature. There are other natural sources that have yet to be discovered that can assist with treating AMR infections. As mentioned earlier, 75% of the current antibiotics are derived from natural products such as local

soil bacteria, plants, and animals. For example, penicillin, a commonly used antibiotic that treats various infections, was isolated from a fungus. Naturally, scientists are exploring new natural products that may have been overlooked to find new antibiotics. To avoid looking in areas where humans populate, scientists have broadened their search for new bacteria that can be used as antibiotics to regions that have not been impacted in areas such as the rainforest and the deep sea.

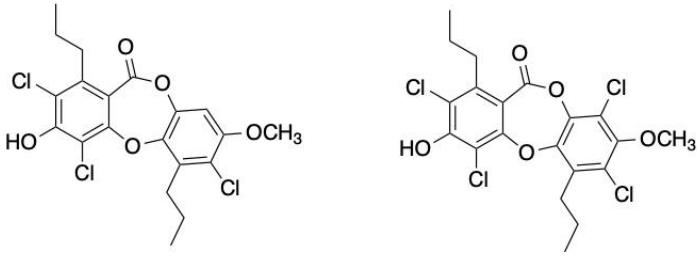
One class of natural products from the deep sea that has piqued the interest of scientists are the depsidones. Depsidones are a family of natural products from lichen, a marine lichen The class definition of depsidones is a 6,7,6-fused ring structure. This compound is known to possess anti-inflammatory and antibacterial properties (**Figure 1**). Depsidones are composed of two phenyl groups that are joined by an ester and ether linkage of a heptacyclic lactone ring in the center. The derivatives of depsidones have varying substituents along the phenyl groups and these groups can be altered in the laboratory to optimize their biological activities.

Figure 1. Unmodified Depsidone Core



Depsidone analogs have even shown to be more active against methicillin-resistant *Staphylococcus aureus,* a known multi-drug resistant species, than other clinically used antibacterial drugs.[4,5] Depsidone's mechanism of antibiotic action against AMR and non-AMR microorganisms is still unknown, which is why scientists are studying the structure and properties of this compound so closely. In 2014, Siwen Nui et al., and other research members from the Laboratory of Natural and Biomimetic Drugs, in Beijing, studied fifteen newly discovered depsidone analogs and their activities. These scientists were able to test the analogs against both Gram-negative bacteria and Gram-positive bacteria. This experiment concluded that the depsidone analogues proved to be effective against the Gram-positive bacteria membranes, which have thick peptidoglycan layer and no outer lipid membrane, inhibiting its growth better than the controls (**Figure 2**).[6] The next step in this experiment would be to modify the derivatives so that they are able to penetrate the double membrane of Gram-negative bacteria so that they can inhibit its growth as well. Gram-negative bacteria have thin, inner layer of peptidoglycan and a rich outer membrane.

Figure 2. (A) The depsidone analog was able to inhibited *Staphylococcus aureus* ATCC 29213 and resulted in a MIC value of 0.5 (µg/mL). **(B)** Similar depsidone analog with an additional chloride. The analogs additional chloride enhanced inhibitory effects against S*taphylococcus aureus* ATCC 29213. MIC value of 0.125 (µg/mL).



In order to take the important steps towards a new targeted drug delivery system, the first would be to synthetically design these compounds in order to analyze the structural activity relationship. The unsubstituted 6,7,6- fused ring system has been extensively examined in the Wolfe laboratory. John Terrell, a research member in Wolfe's lab, developed the synthesis for the core structure of depsidones (**Figure 1**). The focus of Terrell's research was modifying

the ester linkage at the heptacyclic lactone ring to an amine and an amide, examining how this would affect the overall antibacterial activity. Other research members have explored changing the substituents on the phenyl groups to increase their antibiotic activity as well. The results from the prior experiments were that the core structure of the depsidone proved to be essential for its biological activity, which alludes to a more meaningful modification occurring around the outer phenyl groups. Additionally, research members were unable to produce high yields of their product, which is an aspect that will be improved in the current research project.

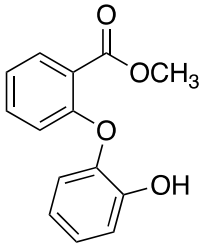
In recently published literature, nanoparticles have been an effective alternative for drug delivery and antibacterial activity.[7,8] Dong, X et al., determined that in the presence of nanoparticles, bacteria become deformed and deflate like a ball that is having the air let out before dying. [8] To replicate a similar effect in *Staphylococcus aureus*, a Gram-positive bacterium, this research will aim to coordinate gold nanoparticles to the depsidone, with the goal of enhancing the overall antibacterial activity. In order to coordinate gold or silver nanoparticles it must first have a binding site on the ligand. Both gold and silver nanoparticles have a strong affinity for thiol groups.[9] Therefore, all depsidone derivatives will have a binding site, which consist of a thiol group, to achieve appropriate binding interactions. This new research will test a series of modified depsidone that are covalently bonded to nanoparticles against both Gram-negative and Gram-positive bacteria. Our group has decided to prioritize testing against Gram-negative bacteria largely due to its unique resistance against antibacterial treatment. The outer membrane of the gram-negative bacteria has special properties that protect it from antibiotics. There are two different approaches that can be taken to penetrate the Gram-negative membrane. The first approach is to increase the size of the depsidone without changing the core structure, which has been proven to be vital for promoting activity. The other approach is to coat gold nanoparticles with the depsidone analog. The nanoparticle should help with delivering the compound to a specific target area, which is through the Gram-negative bacteria outer membrane. Once the derivative arrives within the cell its antimicrobial property should interrupt the cell wall synthesis or inhibit the vital processes that allow Gram-negative and Gram-positive bacteria to replicate. The goal of this project is to create a derivative that is highly effective against both Gram-positive and Gram-negative bacteria.

2. Experimental

General. Reagents and solvents were purchased reagent-grade and used without further purification. Citrate-capped gold nanoparticles were purchased through Fisher Scientific and reactions must be performed in flame-dried glassware under Ar or N_2 atmosphere. Evaporation and concentration *in vacuo* will be performed at 40 °C. TLC and conducted using pre-coated SiO_2 60 F254 glass plates from EMD with visualization by UV light (254 or 366 nm). NMR (^{1}H or ^{13}C) were recorded on an Oxford Varian-400 MHz spectrophotometer at 298 K. Residual solvent peaks were used as an internal reference. Coupling constants (J) (H, H) are given in Hz. Coupling patterns are designated as singlet (s), doublet (d), triplet (t), quadruplet (q), quintuplet (qu), multiplet (m), or broad singlet (br). Low-resolution mass spectral data were acquired on a Shimadzu single quadrupole LCMS-2020. High-resolution mass spectral data were acquired on a Thermo Scientific Q Exactive Plus mass spectrometer coupled to a Waters Acquity UPLC, and the detected masses are given as m/z with m representing the molecular ion. The purity of each tested compound (>95%) was determined via ^{1}H NMR.

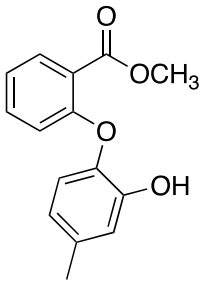
2.1 Preparation for Esterification of Boronic Acid

Methyl 2-(2-hydroxyphenyl) benzoate (I) Mix 2-methoxycarbonylphenylboronic acid (4.929 g, mol), catechol (1.012g), copper acetate (3.264g), pyridine (2.2 mL), and CH_2Cl_2 (30.0 mL) using a 3:1:1:3 molar ratio. Solution was left to stir for one week and then purified via fritted funnel (layered with celite). This step is necessary in order to remove excess copper acetate. Additional purification was performed using flash chromatography. Percent Yield: 22 %



^{1}**H-NMR:** ($CDCl_3$, *-d*, 400MHz) 8.791 (s, 1H), 8.034 (d, 1H), 8.010 (d, 2H), 7.621 (d, 1H), 7.532 (m, 1H), 7.232 (q, 2H), 3.945 (s, 3H), **LCMS:** M+1, 245; M-1, 243.

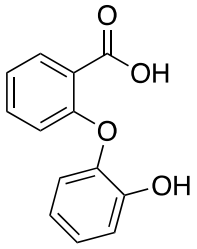
Methyl 2-(2-hydroxy-4-methylphenoxy)benzoate (II) Mix 2-methoxycarbonylphenylboronic acid (4.929 g, mol), 4-methylcatechol (1.06 g), copper acetate (3.264g) , pyridine (2.2 mL), and CH_2Cl_2 (30.0 mL) using a 3:1:1:3 molar ration. Solution was left to stir for 3 days and then purified and filtered through celite. This step is necessary to remove excess copper acetate. Additional purification was performed using flash chromatography (5 % ethyl acetate: 95% hexane). Percent Yield: 18 %



^{1}**H-NMR:** ($CDCl_3$, *-d*, 400MHz) 9.584 (s, 1H), 7.988 (t, 2H), 7.564 (t, 2H), 6.7980 (m, 3H), 3.604 (s, 3H), 2.289 (d, 3H) ppm. **LCMS:** M+1 259; M-1, 257.

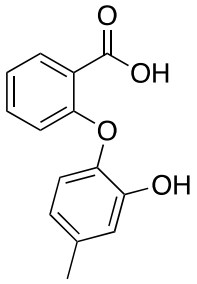
2.2 Preparation for Deprotection of Methyl Ester

2-(2-hydroxyphenoxy)benzoic acid (III) The 4-(Benzyloxy)-3-hydroxybenzoic acid (0.3996 g) was dissolved in a solution of 2M NaOH (1.63g) and 0.1M of methanol (16.36 mL), using a molar ratio of 1:3:1. Solution was stirred for 24 hours. The solution was then acidified with hydrochloric acid (1M) to a pH of 4. Additional purification was performed using flash chromatography (5 % ethyl acetate: 95% hexane). Percent Yield: 54 %



^{1}**H-NMR:** ($CDCl_3$, *-d*, 400MHz) 12.902 (s, 1H), 12.007 (s, 1H), 8.304 (t, 1H),7.827 (t, 1H), 7.481 (t, 2H), 6.868 (m, 4H), 6.133 (d, 1H). **LCMS:** M+1, 231; M-1, 229.

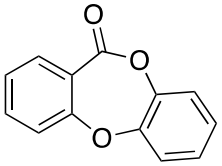
2-(2-hydroxy-4-methylphenoxy)benzoic acid (IV) The methyl 2-(2-hydroxy-4-methylphenoxy)benzoate (0.2266 g) was dissolved in a solution of 2M NaOH (3.7106 g) and 0.1M of methanol (9.27 mL), using a molar ratio of 1:3:1. Solution was stirred for 24 hours. Then the solution was acidified with hydrochloric acid (1M) to a pH of 4. Solution was purified using flash chromatography (5 % ethyl acetate: 95% hexane). Percent Yield: 66%



H-NMR: ($CDCl_3$, *-d*, 400MHz) 11.891(s, 1H), 9.723 (s, 1H), 7.540 (t, 2H), 7.173 (d, 1H), 6.999 (q, 3H), 2.004 (s, 3H) **LCMS:** M+1245; M-1, 243.

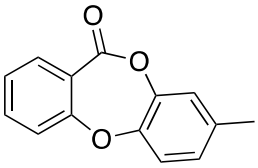
2.3 Preparation of Ring Closure

11*H*-dibenzo[*b*,*e*][1,4]dioxepin-11-one (V) The carboxylic acid (0.01 g) was dissolved in 0.02M of anhydrous THF (0.50mL) along with anhydrous pyridine (1.72 mL) and thionyl chloride (0.25 mL). This reaction was carried out using a 1:20:3 molar ratio in a flame-dried round bottom flask under an inert Ar atmosphere. Then the solution was stirred for 24 hours. The crude reaction was acidified using aqueous hydrochloric acid (1N) to pH < 4 and extracted using ethyl acetate. The crude products were separated and filtered using a flash chromatography.



^{1}**H-NMR:** ($CDCl_3$, *-d*, 400MHz) 8.289 (d, 1H), 7.892 (t, 2H), 7.242 (q, 3H), 7.011 (d, 1H), 5.374 (s, 1H) **LCMS:** M+1, 213; M-1, 211.

8-methyl-11*H*-dibenzo[*b*,*e*][1,4]dioxepin-11-one (VI) The carboxylic acid (0.2787 g) was dissolved in 0.02M of anhydrous THF (0.0026 mL) along with anhydrous pyridine (0.64 mL) and thionyl chloride (0.023mL). This reaction was carried out using a 1:20:3 molar ratio in a flame-dried round bottom flask under an inert Ar atmosphere. Then the solution was stirred for 24 hours. The crude reaction was acidified using aqueous hydrochloric acid (1N) to pH < 4 and extracted using ethyl acetate. The crude products were separated and filtered using a flash chromatography.

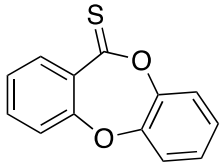


^{1}**H-NMR:** ($CDCl_3$, *-d*, 400MHz) 7.936 (d, 1H), 7.557 (t, 2H), 7.214 (d, 1H), 6.942 (q, 3H), 2.228 (s, 3H) **LCMS:** M+1 227; M-1, 225.

2.4 Procedure Exchange of Ketone for Thioester

11*H*-dibenzo[*b*,*e*][1,4]dioxepine-11-thione (VII) The unmodified depsidone core was dissolved using anhydrous toluene (0.15M) and added to Lawesson's reagent in a 20:13 molar ratio before being transferred into a flame-dried round bottom flask. The reaction was refluxed at 110 ºC in the dark for 20 hours under inert conditions. The reaction was cooled to room temperature before undergoing concentration under reduced pressure. The crude products were

separated using flash column chromatography (SiO_2, 5% ethyl acetate, 95% hexane).



2.5 Ligand Exchange Reaction

The ligand exchange reaction of the new thio ligand, the thioester depsidone, will be performed using previously published literature.[10] The thioester depsidone must be dissolved in CH_2Cl_2.: MeOH (1:1, 4 ml) with citrate coated AuNPs and should be carried out over the course of 5 days at rt (~25 °C). After 5 days, the solvent will be removed under reduced pressure and then washed with organic solvents such as hexanes, then followed by ether. Next, the AuNPs will dissolved in water and then further purified by liquid-liquid extraction using CH_2Cl_2. Nuclear Magnetic Resonance (NMR) will be used to check the purity.

2.5.1 Procedure

30 mg of citrate coated AuNPs will dissolved in 4 mL of purged CH_2Cl_2 and the solution will be left to stir. Additionally, 90 mg of the depsidone ligand must dissolve in 4 mL purged CH_2Cl_2: MeOH (1:1) solution. Then the ligand solution will be slowly added to citrate coated AuNPs while stirring. The solution mixture will then purge with N_2 to remove any trace of CH_2Cl_2. The ligand exchange reaction was then carried out for 3 days under continuous stirring.

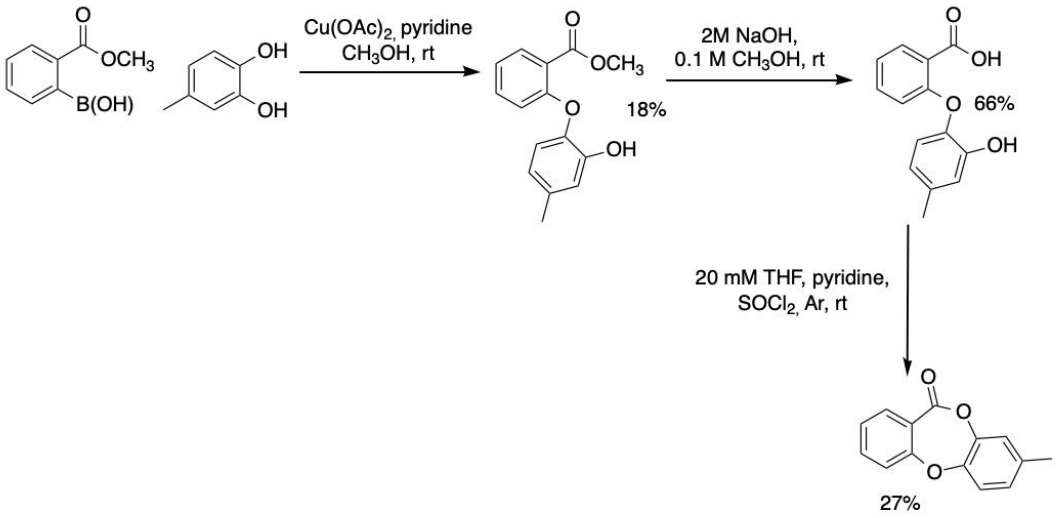
2.6 General Procedure for Testing Compounds via 96- Well Plate Bioassay

Workstation and gloves were sterilized using ethanol. Plate was prepared under aseptic conditions specifically under the flame from a propane torch. Row one of the 96-well master plate contained the following: (A) the depsidone analog dissolve in DMSO 10 µL (B) of the negative control 10 µL (pure DMSO) (C) 10 µL of the antibiotic chloramphenicol as a positive control. Seral dilution of the methylated depsidone was also performed using a multichannel pipette (100mg/mL, 10 mg/mL, 1 mg/mL, 0.1 mg/mL, 0.01mg/mL and 0.001 mg/mL). The serial dilutions were accomplished by taking 1 µL from the top of the well and mixing it into the well below and down each column. The dilutions were then transferred from the master plate to the test plate. The plate was left to incubate overnight at 37 ºC. Absorbance was read for each well on a BioTek plate-reader at 590 nm using
Standard *P. Aeruginosa* Assay Protocall_590nm_blanks.prt and analyzed using Gen5 2.09 software data package.

3. Results and Discussion

The core structure of the depsidone analog was successfully identified and confirmed using NMR as well as Mass spectrometry. The general synthesis is a simple three step process (**Scheme 1**). The procedures used to make depsidone with the addition of a methyl group is fairly like the procedure used to synthesize and unsubstituted core depsidone. However, one of the difficult aspects of this project is the extremely low yield of depsidone analog (~ 5mg). The low yield of products indicates that there is a need for optimization. TLC's results reveled an excess of starting material, to overcome this issue, each step in the general synthesis will react longer than 8 hours to increase a higher yield of desired product rather than starting material.

Scheme 1. Synthetic Scheme of Depsidone Analog



The bioassay results of the methylated depsidone, in *Pseudomonas aeruginosa*, showed a small amount of activity (5 % inhibition). Previous research members, Nutt, C. unpublished results, have tested depsidone analogs against *E. Coli*, a Gram- negative bacteria, and these compounds showed similar activity as well. *Pseudomonas aeruginosa* is one of the more difficult Gram-negative bacteria to treat, therefore little inhibition in this assay was to be expected. Based on the results that the antibacterial potency of the depsidone analog had little to no effect against Gram-negative bacteria investigating slightly longer chains, such as ethyl groups, could improve potency. Additional testing is required to confirm and solidify these claims.

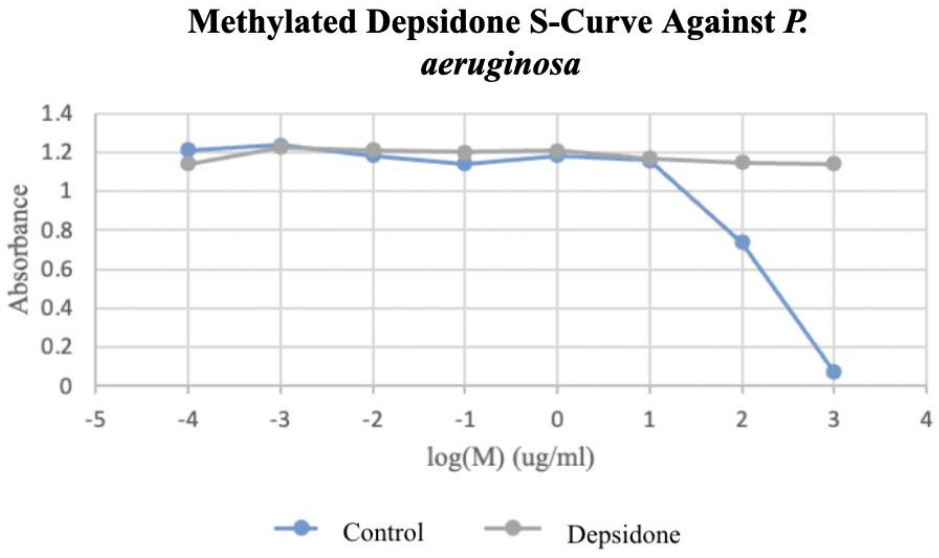


Figure 3: S-Curve of Unmodified Core with methyl substituent Against *P. aeruginosa*

4. Conclusions

As mentioned earlier, isolation of the first product was confirmed through NMR analysis. The next step in this project is to make a substitution at the ester position within the original core structure of the depsidone with a thioketone. Substitution at the ester position rather than altering the side rings, will allow for a greater success with the ligand exchange reaction with the citrate coated gold nanoparticles. After ligand exchange of the thionate depsidone and gold nanoparticles has been successfully identified this compound will be tested against Gram-Negative and Gram-Positive bacteria.

Another approach that this project would continue to explore is the addition of alkyl chains to the outer benzene rings. The idea behind this approach is to explore whether these non-polar groups will improve the experimental inhibition and efficacy of this compound. Previous research in the lab has also supported the idea that alterations to the outer ring to heterocyclic ring results in a decrease in the antibacterial properties of the depsidone. Using alkyl chains will help test whether increasing hydrophobicity of the compound will lead to an increase in potency. Additionally examining steric hindrance on the outer rings has yet to be examined, therefore testing these analogs with steric hindrance made at the ketone position, and on the outer ring could help support the theory that steric hindrance at either position plays a role in its potency against Gram-negative and Gram-positive bacteria.

5. Acknowledgements

I am thankful to Amanda Wolfe for her constant guidance throughout this project. I would also like to extend my appreciation to students who have laid the groundwork and inspired me to expand on this research.

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