EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 5
Issue: 01
63-69
Year: 2018

STRUCTURE-ANTIOXIDANT ACTIVITY RELATIONSHIP IN A SERIES OF SCHIFF BASE COMPOUNDS DERIVED FROM *META*-DIAMINOBENZENE

Kodjo Charles Guillaume*[a, b], Ouattara Zana Adama[a], Yapi Acafou Thierry[c], Kangah Niameke Jean-Baptiste[b], Kablan Richmond Jean-François[d], Konan Dibi Jacques[c] , Kablan Ahmont Landry Claude[e] and Ziao Nahosse[b]

[a]Laboratoire de Chimie BioOrganique et de Substances Naturelles, Université Nangui Abrogoua, UFR-SFA, 02 B.P. 801 Abidjan 02, Côte d'Ivoire,
[b]Laboratoire de Thermodynamique et Physicochimie du Milieu, Université Nangui Abrogoua, Côte d'Ivoire,
[c]Laboratoire de Chimie Organique et de Substances Naturelles, UFR SSMT, Université Félix Houphouet Boigny, 01 BP V34 Abidjan 01, Côte d'Ivoire,
[d]Laboratoire de Cristallographie et Physique Moléculaire, UFR SSMT, Université Félix Houphouët Boigny, 01 BP V34 Abidjan 01, Côte d'Ivoire,
[e]UFR Des Sciences Biologiques, Université Péléforo Gon Coulibaly de Korhogo, BP 1328 Korhogo, Côte d'Ivoire.

***Corresponding Author: Kodjo Charles Guillaume**
Laboratoire de Chimie BioOrganique et de Substances Naturelles, Université Nangui Abrogoua, UFR-SFA, 02 B.P. 801 Abidjan 02, Côte d'Ivoire.

Article Received on 06/11/2017 **Article Revised on 26/11/2017** **Article Accepted on 16/12/2017**

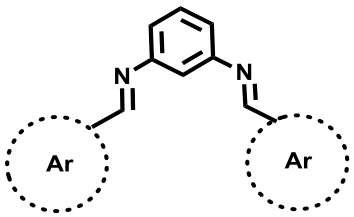
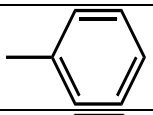
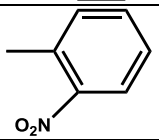
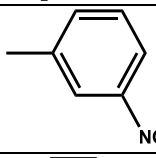
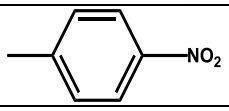
ABSTRACT
Structural modification of the compound 1, *N*,*N'*-bis(benzylidene)benzene-1,3-diamine, by substitution of hydrogen atoms with NO_2 electro-attractor group in *ortho*, *meta* or *para* positions on the benzylidene nuclei, allowed to have a coherent positional isomer series named, compounds 2, 3 and 4. These four Schiff bases compounds have been characterized by conventional spectroscopic methods (NMR, IR and MS). Antioxidant screening carried out according to FRAP and DPPH methods revealed important antiradical properties for compounds 2 and 4 even at low concentrations. This study also revealed that, in this series, antioxidant activities were ranked in decreasing order: 4 > 2 > 1 > 3, whatever the method used. Therefore, both FRAP and DPPH methods agree that, from the compound 1, introduction of a nitro group in this structure in *para* position seemed to be the most sensitive position to increase the antioxidant activity of this phamacophore, whereas the *meta* position on benzylidene nuclei appeared to be unfavorable to the improvement of this biological activity.

KEYWORDS: Schiff base, spectrometry, antioxidant activity; DPPH and FRAP methods.

INTRODUCTION
Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Lot studies have shown that oxidative stress is involved in many diseases as a triggering factor or associated with complications of their evolution. Indeed, it is widely recognized that oxidative stress is at the root of diseases such as cancer, cataract, acute respiratory distress syndrome, pulmonary edema[1], diabetes, Alzheimer's disease, Parkinson's disease, rheumatism, cardiovascular disease[2], infectious diseases such as AIDS[3,4], syphilis[5], renal failure[6,7], malaria and gastric ulcers[8,9] etc.

With this alarming situation of the harmful effects of oxidative stress, it is more than just an emergency that, the scientific community, intensively search for new highly effective antioxidant molecules. Versatile Schiff bases, these condensation products of primary amines with carbonyl compounds, in addition to their wide range of biological activities including antifungal, antibacterial, antimalaria, antiproliferative, antiinflammatory, antiviral, antipyretic properties[10-13], etc. can be a source of new molecules with excellent antioxidant powers. As several authors[14,15], our systematic structural and biological activities research on Schiff bases led us synthesize many diimines. In this work we are interested in structure-activity relationship in a series of schiff base compounds derived from *meta*-diaminobenzene shown in Table 1.

Table 1: Structures of Schiff bases synthesized.

	Compound	Ar
	1	
	2	
	3	
	4	

MATERIAL AND METHODS

Material

Benzaldehyde, 2-nitro-benzaldehyde, 3-nitro-benzaldehyde, 4-nitro-benzaldehyde, and benzene-1,3-diamine were procured from Aldrich and used without further purification. All organic solvents were purchased from Merck and dried before use. Melting points were determined in capillary tube using an MPD Mitamura Riken Kogyo (Japan) electrothermal melting point apparatus and are uncorrected. IR spectra in the range 400-4000 cm^{-1} were obtained on a Bruker-Vector FTIR spectrophotometer, with samples investigated as thin film from CDCl3 solution. The ^{1}H NMR spectra were recorded on a Bruker-Avance-300 spectrometer, operating at 300 MHz. The mass spectra were recorded on a TOF LCT Premier (WATERS) Spectrometer coupled to an HPLC Alliance 2695 chain.

Methods

Synthesis of *N, N'*-bis (phenylmethylene) benzene-1,3-diamine

Benzaldehyde (0.4mmol) and cyclohexane-1,2-diamine (0.2mmol) were dissolved in ether (30 ml). At room temperature, the mixture was stirred for 24 hours to give a yellow precipitate. The precipitate obtained was filtered and recrystallized in ethanol (Rf: 0.87 in benzene/acetone (50;50), yield: 82%, mp: 224.6^{o}C).

Synthesis of *N, N'*-bis (2-nitrophenylmethylene) benzene-1,3-diamine

2-nitrobenzaldehyde (0.4mmol) and cyclohexane-1,2-diamine (0.2mmol) were dissolved in ether (30ml). At room temperature, the mixture was stirred for four days to give a brown precipitate. The precipitate obtained was filtered and recrystallized in ethanol (Rf: 0.84 in benzene/acetone (50;50), yield: 89%, mp 129.6 ^{o}C).

Synthesis of *N, N'*-bis (3-nitrophenylmethylene) benzene-1,3-diamine

3-nitrobenzaldehyde (0.4mmol) and cyclohexane-1,2-diamine (0.2mmol) were dissolved in ether (30 ml). At room temperature, the mixture was stirred for three days to give a pale yellow-orange precipitate. The precipitate obtained was filtered and recrystallized in ethanol (Rf: 0. 83 in benzene/acetone (50;50), yield: 87%, mp 184.6^{o}C).

Synthesis of *N, N'*-bis (4-nitrophenylmethylene) benzene-1,3-diamine

4-nitrobenzaldehyde (0.8mmol) and cyclohexane-1,2-diamine (0.4mmol) were dissolved in ether (30 ml). At room temperature, the mixture was stirred for three days to give a red precipitate. The precipitate obtained was filtered and recrystallized in ethanol (Rf: 0.74 in benzene /acetone (50;50), yield: 80%, mp 185.6^{o}C).

The general synthesis of the four compounds is shown in Figure 2.

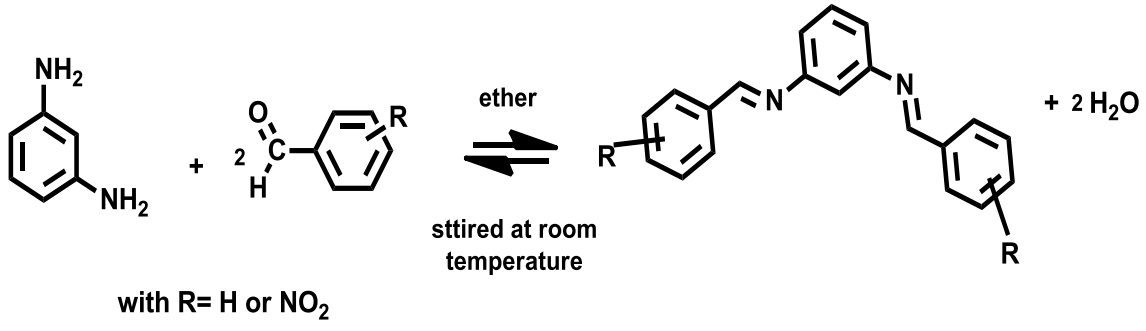


Figure 2: The general synthesis route of compounds 1–4.

Radical scavening test

2,2-diphenyl-1-picrylhydrazyl(DPPH) was one of the first free radicals used to study structure-antioxidant activity relationship of phenolic compounds.[16-18]

*****Principle***

Reduction of the free radical DPPH by an antioxidant can be followed by UV-Visible spectrometry, by measuring the decrease in absorbance at 517nm caused by the antioxidants.[19] In the presence of free radical traps, purple-colored DPPH was reduced to yellow 2,2-diphenyl-1-picrylhydrazine.[20]

*****Dosage***

DPPH radical trapping activity was measured according to the protocol described by Lopes-Lutz et al.[21] and Athamena et al.[22] 100µL of each methanolic solution of the pure compound at different concentrations (0.0625-1 mg / mL) were added to 2.5mL of the methanolic solution of DPPH (0,025g / l). In parallel, a negative control was prepared by mixing 100µl of methanol with 2.5ml of the methanolic solution of DPPH. Absorbance reading was made against a blank prepared for each concentration at 517nm after 30 minutes of incubation in the dark and at room temperature. The positive control was represented by a solution of a standard antioxidant ascorbic acid, whose absorbance was measured under the same conditions as the samples and for each concentration.[23]

The results were expressed in inhibition percentages (I%) of free radical using the following formula.

I% = [(Abs of con neg - Abs sample) / Abs of con neg] x 100

I%: Percentage of DPPH inhibition.

Abs Sample: Absorbance of the sample.

Abs of con neg: Absorbance of negative control

FRAP(Ferric Reducing Antioxidant Power) assay

*****Principle***

The process was based on the reduction of a triazine-tripyridyl ferric complex to ferrous iron in the presence of antioxidants (test sample).

In fact, the sample turns to the blue color followed by colorimetric assay at 593 nm in the presence of antioxidant. The reagent will be prepared as follows.

*****Dosage***

The protocol was based on the method of Benzie et al[24], which had undergone some modifications by Pulido et al.[25] A freshly prepared FRAP solution composed of.

- 25 ml of 300 mM acetate buffer.
- 2.5 ml of 10 mM TPTZ solution in 40 mM HCl solution.
- 2.5 ml of a solution of iron chloride at 20 mM.

The mixture was incubated at 37°C for the duration of the experiment.

The test consisted of mixing in glass hemolysis tubes 100µl of extract diluted with 300µl of distilled water and then with 3000 µl of working solution maintained at 37°C. The absorbance was measured at 593nm after incubating the reaction in a water bath thermostated at 37°C in the dark for exactly 30 minutes.

The calibration line was derived from the absorbance read for the trolox solution range (0.0625 to 1mg/mL) used as antioxidant reference.

The concentration in mg/mL of trolox equivalent per gram of dry matter was calculated based on the regression line of the trolox sampling curve.

RESULTS AND DISCUSSION

Mass Spectra (MS) and IR

Mass spectrum and the infrared spectrum of the synthesized compounds are given in Table 2.

Table 1: Mass spectrum and selected infrared data.

Compound	Mass spectrum $[M+H]^+$ (g/mol)	infrared spectrum: (Cm^{-1})	
		($\upsilon_{C=N}$)	(υ_{C-H})
1	285	1629	3000-2770
2	375	1661	3000-2750
3	375	1656	3000-2790
4	375	1660	3000-2750

*****MS study***

The mass spectra (HR-ESI-MS) of the title compounds showed peaks corresponding to the molecular ions at m/z 375 $[M + H]^+$, that allowed to propose $C_{20}H_{14}N_4O_4$ empirical formula for compounds 2, 3 and 4. Concerning compounds 1 the peak at m/z 285 $[M + H]^+$, was conform to propose $C_{20}H_{16}N_2$ empirical formula.

*****IR study***

The IR spectra showed characteristic bands at 1629 cm^{-1} for compound 1, 1661 cm^{-1} for compound 2, 1656 cm^{-1} for compound 3 and 1660 cm^{-1} for compound 4. These bands corresponded to the elongation vibration of the two azomethine vibrators C = N present in each molecule structure. Thus, the fact of obtaining only one vibration band $\upsilon_{C=N}$ for the two C=N bonds attested that the molecules studied were symmetric. The absence of N-H vibrator bands around 3500cm^{-1} on the spectra confirmed the absence of amine in synthesized products. The multi-bands located between 3000 cm^{-1} and 2750cm^{-1} indicated in Table 2, correspond to υ_{C-H} elongation vibrations of both benzene nuclei and cyclohexane fragments.

^{1}H NMR Spectroscopy

^{1}H NMR spectral data in deturated CDCl3 solution of the synthesized compounds are given in Table 2.The resonance of protons had been assigned on the basis of their integration and multiplicity pattern.[26] The ^{1}H NMR spectra exhibited signals at 8.90; 8.93; 8.92; 8.90 ppm for compounds 1, 2, 3 and 4, attributed to iminic protons C<u>H</u>=N-, respectively. The multi-signals within the 8.77-7.22ppm range are assigned to the aromatic protons of the three rings.

Table 2: ^{1}H NMR data [a-c] of compounds

Compound	Molecular formula	N=C<u>H</u> (s)	$C_6\underline{H}_{4(5)}$ (m)
1	$C_{20}H_{16}N_2$	8.90;(2H)	8.75-7.32;(14H)
2	$C_{20}H_{14}N_4O_4$	8,93;(2H)	8.19-7,22;(12H)
3	$C_{20}H_{14}N_4O_4$	8.92 ;(2H)	8.77-7.35;(12H)
4	$C_{20}H_{14}N_4O_4$	8.90 ;(2H)	8.38-7.30;(12H)

a Multiplicity is given as s = singlet, m = multi-signals
b Chemical shits in ppm
c Integration: number of protons in brackets

The ^{1}H-NMR spectral data of the Schiff bases synthesized were in accord with the proposed structures.

Ferric Reducing Antioxidant Power (FRAP) Assay

The results of the ferric reduction antioxidant power, expressed in mg/mL Trolox equivalent, obtained from a calibration straight line at different concentrations, are presented in Table 3.

Table 3: Antioxidant power of ferric reduction of pure compounds.

Compound	FRAP value
3	0,362432 ±0,084319 (**)
1	0,557220 ± 0,125034 (**)(***)
2	0,616938 ± 0,128615 (***)
4	0,648860 ±0,151572 (***)
Fisher value F	11,727
P value	< 0,001

Compounds with the same symbol (*) have their activities not very different from each other
(***): Best activity; (**): Average activity;(*): Low activity

Statistical Analysis: *Data were expressed as mean ± standard deviation. n = 3 (test numbers). The influence of the different concentrations on inhibition percentage was studied by comparison of means through variance analysis (ANOVA). P<0.05*[27] *was considered as significant. Statistical analysis was done using Microsoft Office Excel-2007 and SAS 9.1software.*

According to FRAP method, in this series, antioxidant activities were ranked in as follow decreasing order: 4 > 2 > 1 > 3. These results revealed that the compounds 4 and 2 with FRAP values respectively of 0.648860 ± 0.151572 and 0.616938 ± 0.128615, were the most active compared to compound 1 (p <0.001). It is deduced that the *Para*- nitro substituted compound 4 was the very most active of them. Compound 3 with its FRAP value of 0.557220 ± 0.125034 was the least active of them. The compound 1 meanwhile, had mean antioxidant activity. From the compound 1, introduction of a nitro group in this structure sensibly increased the antioxidant activity of the compound 4 and sensibly decreased this activity for compound 3. Therefore, *para* position on the benzylidene nuclei seemed to be the most sensitive position which promotes biological activity.

Anti-radical activity by DPPH method

Statistical analysis in this study gave the results recorded in Table 4. Analysis of Table 4 showed that all the compounds studied had antioxidant properties. We also observed a significant difference (P <0.001) between inhibition percentages when switching from one compound to another. Inhibition percentage determination indicated 71.559 ± 5.972; 68.896 ± 7.540; 65.487 ± 6.007; 51.109 ± 5.535; 41,696 ± 5,919, respectively for compound 4, ascorbic acid, compounds 2, 1 and 3. Like FRAP method, DPPH assays showed that the antioxidant activities were ranked one more time in 4 > 2 > ascorbic acid > 1 > 3 decreasing order. For molecules 1 and 3, we obtained relatively lower inhibition values than ascorbic acid, but with a non-negligible inhibition effect.

Table 4: Inhibition percentage values by DPPH method.

Compound	FRAP value
3	41,696 ± 5,919 (***)
1	51,109 ± 5,535 (***)
2	65,487 ± 6,007 (****)
Ascorbic acid	68,896 ± 7,540(****)
4	71,559 ± 5,972(****)
Fisher value F	34,2852
P value	< 0,001

Compounds with the same symbol (*) have their activities not very different from each other
(***): Best activity; (**): Average activity; (*): Low activity

Statistically, compound 2 had the same antiradical effect as ascorbic acid. Compound 4 was the one that most strongly inhibited DPPH even at low concentrations, as shown by the histograms below.

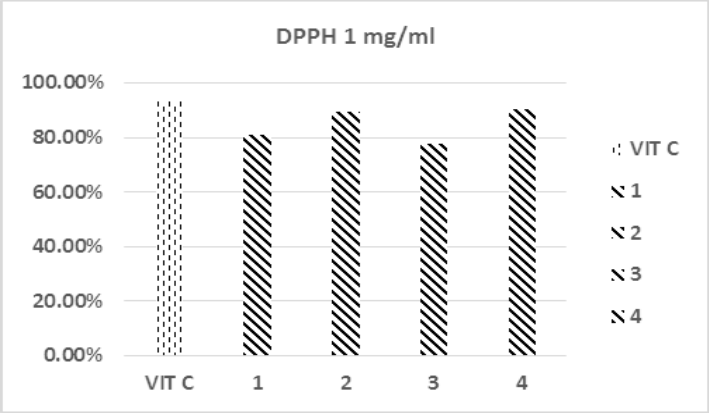


Figure 3: Antioxidant activity by DPPH method at 1mg/ml.

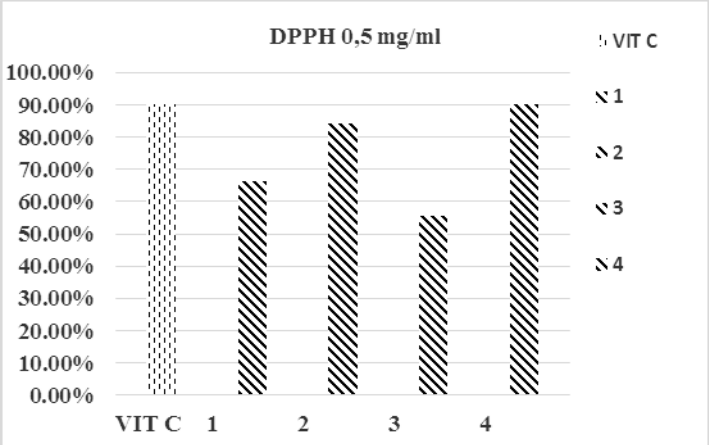


Figure 4: Antioxidant activity by DPPH method at 0.5 mg/Ml.

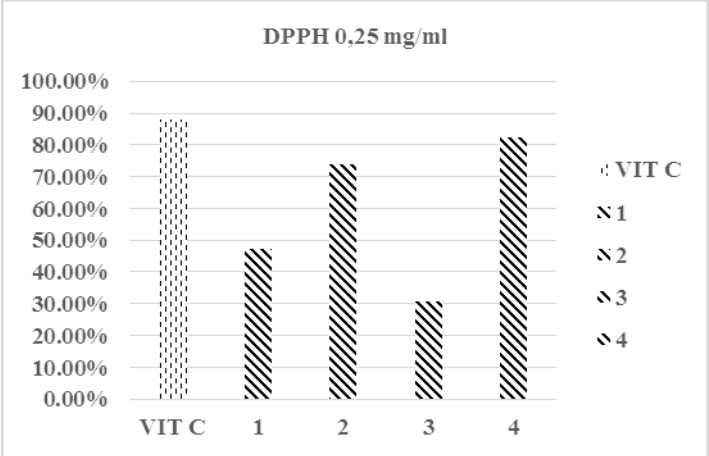


Figure 5: Antioxidant activity by DPPH method at 0.25 mg/mL.

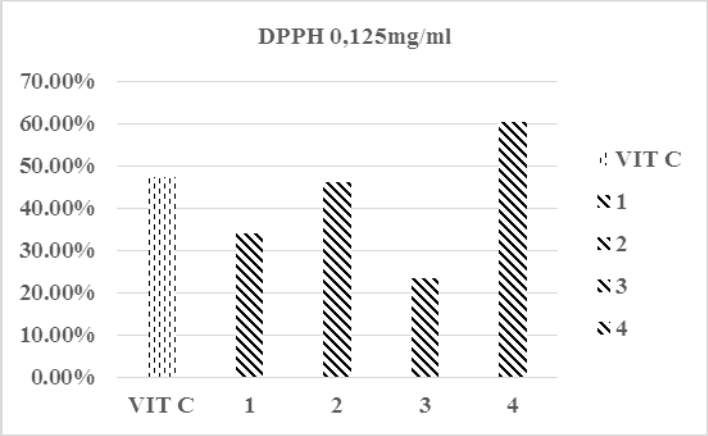


Figure 6: Antioxidant activity by DPPH method at 0.125 mg/mL.

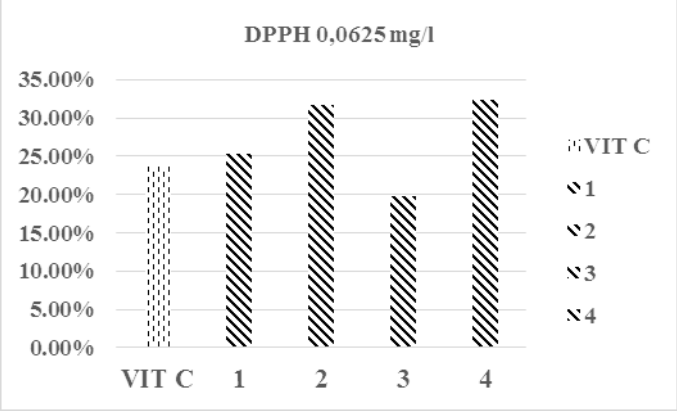


Figure 7: Antioxidant activity by DPPH method at 0.0625 mg/mL.

These histograms revealed in detail the antioxidant power of this series of compounds. In the concentration range localized between 1 and 0.25mg/ml, the inhibition percentage values are comparable to ascorbic acid one. On the other hand, at 0.125mg/ml and 0.0625mg/ml, some of these compounds had a greater antiradical effect than vitamin C. At 0.125mg/ml, compound **4** was the only one that strongly inhibited DPPH radical and showed an antiradical activity greater than the reference molecule. At 0.0625mg/ml almost all the compounds were much more active than ascorbic acid except *meta* substituted compound 3. Anyway, whatever the concentration, from *N*, *N'*-bis (benzylidene) benzene-1,3-diamine compound, substitution of the electro-attractor nitro group in *meta* position on benzylidene nuclei seemed to be unfavorable to improve the biological activity. Whereas, *ortho* position and, even more, *para* position appeared to be the sites most sensitive to increase the antioxidant activity of this phamacophore. For this class of molecule, it is the first time, to our knowledge, that, such important antioxidant properties were observed.

CONCLUSION

Antioxidant screening carried out according to FRAP and DPPH methods revealed important antiradical properties for compounds 2 and 4. This study also revealed that the antioxidant activities were arranged in decreasing order: 4 > 2 > 1 > 3. *Para*-substituted compound 4 which was in all cases the most active, exhibited a much higher antioxidant activity than the reference compound (ascorbic acid) even at low concentrations. With its excellent antioxidant activity, compound 4 had proven to be a good candidate to become a lead compound for the development of a new class of Schiff bases profile antioxidant compounds.

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