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Synthesis, characterization and *in vitro* inhibition of metal complexes of pyrazole based sulfonamide on human erythrocyte carbonic anhydrase isozymes I and II

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ABSTRACT

Sulfonamides represent an important class of biologically active compounds. A sulfonamide possessing carbonic anhydrase (CA) inhibitory properties obtained from a pyrazole based sulfonamide, ethyl 1-(3-nitrophenyl)-5-phenyl-3-((5-sulfamoyl-1,3,4-thiadiazol-2-yl)carbamoyl)-1*H*-pyrazole-4-carboxylate (**1**), and its metal complexes with the Ni(II) for (**2**), Cu(II) for (**3**) and Zn(II) for (**4**) have been synthesized. The structures of metal complexes (**2**–**4**) were established on the basis of their elemental analysis, ^{1}H NMR, IR, UV–Vis and MS spectral data. The inhibition of two human carbonic anhydrase (hCA, EC 4.2.1.1) isoenzymes I and II, with **1** and synthesized complexes (**2**–**4**) and acetazolamide (AAZ) as a control compound was investigated *in vitro* by using the hydratase and esterase assays. The complexes **2**, **3** and **4** showed inhibition constant in the range 0.1460–0.3930 μM for hCA-I and 0.0740–0.0980 μM for hCA-II, and they had effective more inhibitory activity on hCA-I and hCA-II than corresponding free ligand **1** and than AAZ.

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Carbonic anhydrase; hydratase and esterase activities; metal complexes; pyrazole; sulfonamide

Introduction

Sulfonamides represent a significant class of biologically active compounds that inhibit carbonic anhydrase (CA, EC 4.2.1.1) isoenzymes involved in different pathological and physiological events. Carbonic anhydrases are a super family of metallo enzymes that catalyze the interconversion of carbondioxide (CO_2) and water (H_2O) to bicarbonate (HCO_3^-) and proton (H^+)[1–6].

The α-CA is the best known group in the six genetically distinct CA families (α-, β-, γ-, δ-, ε-, ζ- and η-)[7–13]. A total of 16 isoenzymes have been previously described as members of the α-CA family, and classified according to their subcellular localization. CAs I, II, III, VII and XIII are cytosolic isoenzymes[14–16]. CAs VA and VB are localized in the mitochondria[17–19], CA VI is a unique secreted isoenzyme[18,19] CAs IX, XII and XIV are transmembrane proteins[20,21] and CAs IV and XV are GPI-anchored to the cell membrane.[22]

1,3,4-Thiadiazole-2-sulfonamide derivatives[23–28] played a critical role in the development of several important classes of pharmacological agents, such as the diuretics with saluretic action,[29,30] benzothiadiazine[31] and high-ceiling diuretics,[32] or the antiglaucoma drugs with CA inhibitory action, among others.[33,34]

The coordination chemistry of pyrazoles and other heterocyclic compounds containing nitrogen has attracted much attention because of their interesting structural properties and application in diverse areas.[35] The ease of synthesis of various substituted pyrazoles is the most interesting feature in the incorporation of pyrazole groups in the design of new ligands and offers the opportunity of both electronic and steric control of the properties of the metal complexes.

Generally, metal complexes of heterocyclic sulfonamides are 10–100 times more active as CA inhibitors than the sulfonamides from which they were obtained, assuring affinities for the receptor in the 10^{10} to 10^{12} range.[36–41]

Recently, we have reported the metal complexes of some pyrazole-based 1,3,4-thiadiazole-2-sulfonamides show very strong CA inhibitory properties.[42,43]

In this work, three new air stable complexes **2**, **3** and **4** have been reported, and they can be seen as candidates for further *in vivo* studies for the treatment of glaucoma which were synthesized by the reaction of M(II) (Ni(II) (**2**), Cu(II) (**3**) and Zn(II) (**4**)) with **1** (Figure 1). The newly obtained complexes **2**, **3** and **4** were characterized by standard procedures in order to assign their structures, and were assayed as CA inhibitors against hCA-I and hCA-II isoenzymes.

Materials and methods

All reagents were the highest grade commercially available and used without further purification. Compound **1** was synthesised by literature method.[44] ^{1}H NMR spectrum was recorded with a Bruker DPX FT NMR (500 MHz) spectrometer (Billerica, MA). Elemental analyses for C, H, N and S were performed on a Leco CHNS-932 instrument (St Joseph, MI). MS spectra of the complexes were recorded using by Varian Mat III 80 eV (Palo Alto, CA). IR spectra were recorded on a Bruker Optics, vertex 70 FT-IR spectrometer using ATR techniques. The UV–vis spectra were carried out with a Shimadzu UV-2550 spectrometer (Kyoto, Japan) in the range 900–200 nm. Magnetic susceptibility measurements at room temperature were taken using a Sherwood Scientific Magway MSB MK1 model (Cambridge, UK)

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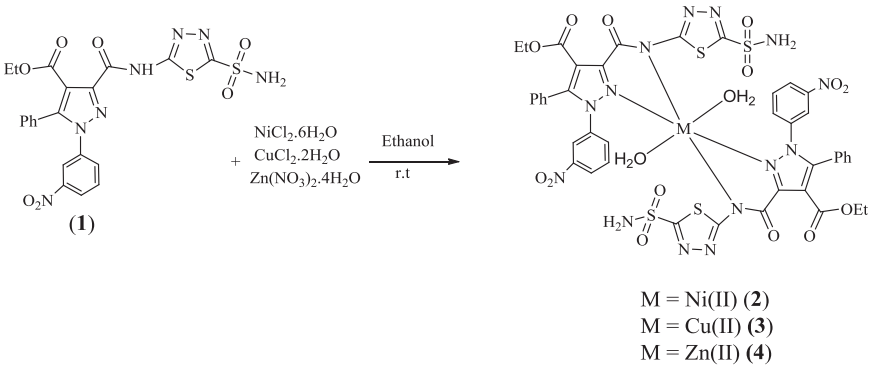

Figure 1. Syntheses of complexes 2, 3 and 4.

magnetic balance by the Gouy method using $Hg[Co(SCN)_4]$ as calibrant.

General procedure for synthesis of 2, 3 and 4

A solution of $MX_2.nH_2O$ ($NiCl_2.6H_2O$ for (**2**), $CuCl_2.2H_2O$ for (**3**) and $Zn(NO_3)_2.4H_2O$ for (**4**)) (2.20×10^{-4} mol) in 10 mL ethanol was added dropwise with continuous stirring to 20 mL of an ethanolic solution containing **1** (1.1×10^{-4} mol). The pH of the reaction mixture was adjusted by addition of 0.1 M aqueous NaOH solution in the range between 6.0 and 7.0. The reaction mixture was stirred overnight at room temperature to give an air stable solid of titled complexes **2**, **3** and **4**.

Synthesis of ***2****:* Blue solid, 67% yield, m.p. 238 °C (decompose); IR (cm^{-1}): 3504 (OH), 3390 and 3235 (NH_2), 3098 $(CH)_{ar.}$, 2984 and 2878 $(CH)_{aliph.}$, 1726 (ester C=O), 1617 (amide C=O), 1536, 1491 (Ar C=C/C=N), 1172 (SO_2), 591 (Ni–O) and 484 (Ni–N).

UV–Vis, [λ (nm), ε_{max} (L mol^{-1} cm^{-1})]: 290 (36 130), 301 (43 400), 304 (43 400) (π–π^*), 784(120) (d–d).

MS: *m/z* 1142.9 $[NiC_{42}H_{32}N_{14}O_{14}S_4]^+$, 544.0 $[C_{21}H_{17}N_7O_7S_2]^+$, 498.0 $[C_{21}H_{18}N_6O_5S_2]^+$, 79.1 $[H_2NO_2S]^+$.

$C_{42}H_{36}NiN_{14}O_{16}S_4$; Anal. calcd (%): C, 42.76; H, 3.08; N, 16.62; S, 10.87. Found (%): C, 42.41; H, 2.89; N, 16.35; S, 10.73.

Synthesis of ***3****:* Green solid, 78% yield, m.p. 224 °C (decompose); IR (cm^{-1}): 3600 (OH), 3412 and 3241 (NH_2), 3094 $(CH)_{ar.}$, 2983 and 2875 $(CH)_{aliph.}$, 1729 (ester C=O), 1624 (amide C=O), 1534, 1489 (Ar C=C/C=N), 1173 (SO_2), 589 (Cu–O) and 472 (Cu–N).

UV–Vis, [λ (nm), ε_{max} (L mol^{-1} cm^{-1})]: 290 (36 700), 301 (43 400) (π–π^*), 780 (250) (d–d).

MS: *m/z* 1147.7 $[CuC_{42}H_{32}N_{14}O_{14}S_4]^+$, 544.0 $[C_{21}H_{17}N_7O_7S_2]^+$, 497.9 $[C_{21}H_{18}N_6O_5S_2]^+$, 79.2 $[H_2NO_2S]^+$.

$C_{42}H_{36}CuN_{14}O_{16}S_4$; Anal. calcd (%): C, 42.58; H, 3.06; N, 16.55; S, 10.83. Found (%): C, 42.65; H, 3.11; N, 16.62; S, 10.71.

Synthesis of ***4****:* White solid, 54% yield, m.p. 239 °C (decompose); ^1H NMR (500 MHz, DMSO-d_6): δ 8.06 (s, 4H, SO_2NH_2), 7.99–7.25 (m, 18H, ArH), 4.11 (s, 4H, CH_2), 1.07 (s, 4H, H_2O), 1.05 (m, 6H, CH_3). IR (cm^{-1}): 3441 (OH), 3397 and 3276 (NH_2), 3074 $(CH)_{ar.}$, 2985 and 2876 $(CH)_{aliph.}$, 1727 (ester C=O), 1606 (amide C=O), 1549, 1473 (Ar C=C/C=N), 1172 (SO_2), 586 (Zn–O) and 478 (Zn–N).

UV–Vis, [λ (nm), ε_{max} (L mol^{-1} cm^{-1})]: 282 (16 260), 323 (10 180) (π–π^*). MS: *m/z* 1148.8 $[ZnC_{42}H_{32}N_{14}O_{14}S_4]^+$, 543.9 $[C_{21}H_{17}N_7O_7S_2]^+$, 498.0 $[C_{21}H_{18}N_6O_5S_2]^+$, 79.2 $[H_2NO_2S]^+$.

$C_{42}H_{36}N_{14}ZnO_{16}S_4$; Anal. calcd (%): C, 42.52; H, 3.06; N, 16.53; S, 10.81. Found (%): C, 43.73; H, 2.86; N, 17.35; S, 11.32.

Purification of isoenzymes hCA-I and hCA-II from human erythrocytes

In order to purify hCA-I and hCA-II isoenzymes, first, human blood was centrifuged at 1500 rpm for 20 min, and after the removal of the plasma, the erythrocytes were washed with an isotonic solution (0.9% NaCl). After that, the erythrocytes were lysed with 1.5 volume of ice-cold water. The lysate was centrifuged at 20 000 rpm for 30 min to remove cell membranes and non-lysed cells. The pH of the supernatant was adjusted to 8.7 with tris and was then loaded onto an affinity column containing Sepharose-4B-L-tyrosine-p-aminobenzene sulfonamide as the binding group. After extensive washing with 25 mM tris–HCl/22 mM Na_2SO_4 (pH 8.7), the hCA-I and hCA-II isoenzymes were eluted with 1.0 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3COONa/0.5 M $NaClO_4$ (pH 5.6).[45,46] The amount of purified protein was estimated by the Bradford method[47] and SDS-PAGE was carried out to determine whether the elute containing the enzyme[48] (Supplemental Figure 1).

Hydratase activity assay

Carbonic anhydrase activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson.[49] CO_2-hydratase activity as an enzyme unit (EU) was calculated by using the equation $((t_0-t_c)/t_c)$ where t_0 and t_c are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively. IC_{50} values (the concentration of inhibitor producing a 50% inhibition of CA activity) have been obtained as *in vitro* for the free ligand (**1**), newly synthesized compounds (**2**–**4**) and **AAZ** as the control compound.

Esterase activity assay

Carbonic anhydrase activity was assayed by following the change in absorbance at 348 nm of 4-nitrophenylacetate (NPA) to 4-nitrophenylate ion over a period of 3 min at 25 °C using a spectrophotometer (CHEBIOS UV-VIS, Roma, Italy) according to the method described in the literature.[50,51] The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL of 0.05 M tris–SO_4 buffer (pH 7.4), 1 mL of 3 mM 4-nitrophenylacetate, 0.5 mL H_2O and 0.1 mL enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution. IC_{50} values have been obtained as *in vitro* for free ligand (**1**), the novel synthesized compounds (**2**–**4**) and **AAZ** as the control compound.

Table 1. IR spectral data (cm^{-1}) of the free ligand (1) and complexes 2, 3 and 4.

Assignments	1	2	3	4
ν(OH)	–	3504 (br)	3600 (br)	3441 (br)
$\nu(NH_2)_{as}$	–	3390 (w)	3412 (w)	3397 (w)
$\nu(NH_2)_s$	3227 (w)	3235 (w)	3241(w)	3276 (w)
$\nu(C–H)_{ar}$	3094 (w)	3098 (w)	3094 (w)	3074 (w)
$\nu(C=O)_{ester}$	1738 (s)	1726 (s)	1729 (s)	1727 (s)
$\nu(C=O)_{amide}$	1659 (s)	1617 (s)	1624 (s)	1606 (s)
ν(C=C/C=N)	1523, 1478, 1431 (m)	1536, 1491 (m)	1534, 1489 (m)	1549, 1473 (m)
$\nu(SO_2)$	1165 (m)	1172 (m)	1173 (m)	1172 (m)
ν(M–O)	–	591 (m)	589 (m)	586 (m)
ν(M–N)	–	484 (m)	472 (m)	478 (m)

w: weak; br: broad; m: medium; s: strong.

Determination of K_i values

The method for determination of K_i values is described elsewhere.[52–56] In the first part of this study, IC_{50}values have been obtained as *in vitro*. IC_{50} of the free ligand (**1**), synthesized complexes (**2**–**4**) and **AAZ** as the control compound were assayed by the hydrolysis of *p*-nitrophenylacetate on esterase activities of CA isoenzymes in the presence of various inhibitor concentrations. The absorbance was determined at 348 nm after 3 min.[52] Regression analysis graphs were drawn by plotting inhibitor concentrations versus enzyme activity by using Microsoft Excel Program.

In the second part of the study, enzyme activity was measured in the presence of five different substrate concentrations at each of these inhibitor concentrations (30%, 50% and 70%), and the data were linearized with Lineweaver–Burk plot in order to obtain K_i values.

^{1}H NMR spectrum of Zn(II) complex (4)

Due to its diamagnetic nature, the Zn(II) complex (**4**) was studied by NMR spectroscopy and its ^{1}H NMR spectrum is given in Supplemental Figure 2. The comparison of the ^{1}H NMR spectrum of the complex (**4**) with the spectrum of free ligand[44] gave valuable information regarding the coordination mode of ligand during complexation. The peak corresponds to CONH proton found in the spectrum of free ligand[44] at δ 12.95 ppm was absent in the spectrum of **4** indicating that the deprotonated CONH group of nitrogen atom participates in the coordination. In free ligand, SO_2NH_2 protons give a signal at δ 8.32 ppm which is shifted to δ 8.06 ppm in Zn(II) complex. In the NMR sectrum of complex **4,** the signals due to aromatic protons are observed at δ 7.99–7.25 ppm range. Upfield shifts of these protons in comparison of the ligand[44] (δ 8.11–7.29 ppm) confirm the coordination of the free ligand to zinc atom. The signals correspond to CH_3 and CH_2 are observed at δ 1.05 and 4.11 ppm, respectively. The singlet with an intensity of four is found at δ 1.07 ppm which assigned as coordinated oxygen atoms of two water molecules to zinc ion.

FT-IR measurements

The significant frequencies in the IR spectra of the free ligand[44] (**1**) and the complexes (**2**–**4**) are given in Table 1. The broad absorption bands at 3504, 3600 and 3441 cm^{-1} are attributed to (O–H) of water in compounds **2**, **3** and **4** respectively. Two stretching frequencies were obtained for the asymmetric (3390 cm^{-1}) and symmetric (3235 cm^{-1}) vibrations of the sulfonamido NH_2 moiety for compound **2**. These asymmetric and symmetric bands were observed at 3412 and 3241 cm^{-1}, respectively, for compound **3** and at 3397 and 3276 cm^{-1}, respectively, for compound **4**. The N–H stretching vibration was observed at 3227 cm^{-1} for **1**. The band corresponding to the amide ν(C=O) vibration of the pyrazole carboxamide (1659 cm^{-1}) of **1** is observed as a single band shifted to lower frequencies (about 1624–1606 cm^{-1}) in the complexes (**2**–**4**). This fact could be attributed to the bonding of the metal ions with the amide nitrogen of the ligand.[57] Absorptions at 1523, 1478 and 1431 cm^{-1} assigned to the ν(C=C/C=N) vibrations of **1** are slightly shifted from those found in **2** (1536 and 1491 cm^{-1}), **3** (1534, 1489 cm^{-1}) and **4** (1549, 1473 cm^{-1}). These data of complexes **2**, **3** and **4** are also attributed to the coordination occurring from nitrogen atom of pyrazole ring of **1**. Weak bands at 591 and 484 cm^{-1} for **2**, 589 and 472 cm^{-1} for **3** and 586 and 478 cm^{-1} for **4** are corresponded with M–O and M–N vibrations, respectively.

UV/vis spectra and magnetic susceptibility

The electronic spectra of compounds **1**–**4** were recorded in DMSO solutions at a 1×10^{-3} M concentration at room temperature (Table 2). The electronic spectrum of **2** exhibits three intense absorption bands at 290, 301 and 304 nm that were assigned to π–π^* and the broad absorption band at 784 nm was assigned to d–d transitions of a 6-coordinate octahedral geometry.[58] Two bands for compound **3** (290 and 301 nm) were assigned to π–π^* transitions similarly to other sulfonamide derivatives containing 1,3,4-thiadiazole-2-sulfonamide ring.[59] In addition, the electronic spectrum of **3** also exhibiting a broad band at 780 nm is attributable to d–d transition. The Zn(II) complex (**4**) did not show any d–d transition and its spectrum was dominated only by the π–π^* transition bands at 282 and 323 nm. The magnetic susceptibility measurements were carried out on powdered samples at room temperature. The magnetic moment value for Ni(II) complex (**2**) is 3.64 B.M. which is in good agreement with a d^8 system in an octahedral environment. The magnetic moments of the complexes at room temperature lie in the range of 3.53–4.26 B.M. for mono nuclear octahedral Ni(II) centers.[60] The measured magnetic moment of **3** is 1.87 B.M. which is consistent with the expected spin-only magnetic moment of d^9 Cu(II) systems. The spectral data and magnetic moment value indicate an octahedral geometry around Cu(II) ion.[61,62] As expected, the room temperature magnetic moment value of Zn(II) complex (**4**) is found to be diamagnetic due to non-availability of unpaired electrons.

MS spectral studies of 2, 3 and 4

The MS spectra give additional structural information about the chemical structure of the studied complexes **2**, **3** and **4**. None of the MS spectra of complexes showed a molecular ion $[M]^+$ peak. The fragment $[MC_{42}H_{32}N_{14}O_{14}S_4]^+$ which suggests the monomeric nature of the complexes is observed at *m/z* 1142.9 for **2** and

1147.7 for **3** and 1148.8 for **4**. The fragment $[C_{21}H_{17}N_7O_7S_2]^+$ is observed at *m/z* 544.0 for **2** and for **3** and 543.9 for **4**. The peaks are observed at *m/z* 498.0 and 79.1 for **2**, 497.9 and 79.2 for **3**, and 498.0 and 79.2 for **4** which are due to the fragment $[C_{21}H_{18}N_6O_5S_2]^+$ and $[H_2NO_2S_2]^+$, respectively. These data confirmed the proposed formula of the complexes (Figure 1).

In vitro *inhibition studies*

Inhibition effects on hCA-I and hCA-II isoenzymes of the newly synthesized compounds (**2**–**4**) and acetazolamide (**AAZ**) as a control compound were studied by hydratase and esterase activity methods and K_i values were determined for each compound and compared to inhibition effect of the free ligand (**1**) (Table 3).

According to the *in vitro* studies, the IC_{50} values of hydratase activities of newly synthesized compounds **3** and **4** (0.0600 and 0.0520 μM for hCA-I and 0.0340 and 0.0420 μM for hCA-II, respectively) are lower than the IC_{50} values of **1** (1.4000 and 0.9000 μM for hCA-I and hCA-II, respectively) and of **AAZ** (3.3000 and 2.4000 μM for hCA-I and II, respectively). The IC_{50} values of hydratase activities of **2** 2.4000 μM for hCA-I is lower than **AAZ** but higher than **1** and 0.0625 μM for hCA-II lower than both **1** and **AAZ**. The IC_{50} values of esterase activities of compounds **2**, **3** and **4** (0.0820, 0.1450 and 0.3400 μM for hCA-I and 0.0190, 0.0900 and 0.0920 for hCA-II) are all lower than the IC_{50} values of esterase activities for free ligand (**1**) (2.8000 and 5.6000 μM for hCA-I and hCA-II, respectively) and **AAZ** (4.6000 and 3.9000 μM for hCA-II, respectively).

In relation to the esterase activities, the inhibition equilibrium constants (K_i) were also determined. The pyrazole carboxamide group of **1** forms a hydrogen bond with the histidine residue in the active site of CA to inhibit the isozymes (K_i: 1.1000 and 5.3000 μM for hCA-I and hCA-II, respectively), resulting in less movement of the carbonic acid toward CO_2 production.[63] The coordination compounds (**2**, **3** and **4**) show remarkable inhibition on hCA-I and hCA-II (K_i: 0.1460, 0.1480 and 0.3930 μM and 0.0960, 0.0724 and 0.0980 μM, respectively), having a higher inhibition as compared to **1** as well as to the control compound **AAZ** (2.8000 and 2.1000 μM for hCA I and hCA-II, respectively), which is probably due to complexation of metal(II) ions to ligand.[64]

In summary, these new compounds (**2**–**4**) have quite a lot higher potential inhibitory effects than their parent inhibitors **1** and **AAZ**. Thus, the complexes (**2**–**4**) might be good candidates for further clinical studies of glaucoma treatment. Especially, compounds **3** and **4** have shown remarkable inhibition against hCA-II and hCA-I isoenzymes for hydratase activities, and compound **2** has shown remarkable inhibition against hCA-I and hCA-II isoenzymes for esterase activities. The order of the metal ions for the inhibition of hCA-I for hydratase activity is Zn > Cu > Ni, and of hCA-I for esterase activity is Ni > Cu > Zn, and of hCA-II for hydratase activity is Cu > Zn > Ni, and of hCA-II for esterase activity is Ni > Cu > Zn.

Table 2. Optical properties of compounds **1**–**4** in dimethylsulfoxide.

	λ_{max} (nm) (ε (L mol^{-1} cm^{-1}))			
	1	**2**	**3**	**4**
DMSO	290 (34 750) 296 (40 390)	290 (36 130) 301 (43 400) 304 (43 400) 784 (120)	290 (36 700) 301 (43 400) 780 (250)	282 (16 260) 323 (10 180)

Table 3. IC_{50} values of **AAZ** and **1**–**4** on hydratase and esterase activity of hCA-I and hCA-II and K_i values of hCA-I and hCA-II.

	Hydratase IC_{50} (μM)		Esterase IC_{50} (μM)		K_i (μM)	
Inhibitor	hCA-I	hCA-II	hCA-I	hCA-II	hCA-I	hCA-II
AAZ	3.3000	2.4000	4.6000	3.9000	2.8000	2.1000
1	1.4000	0.9000	2.8000	5.6000	1.1000	5.3000
2	2.4000	0.0625	0.0820	0.0190	0.1460	0.0960
3	0.0600	0.0340	0.1450	0.0900	0.1480	0.0724
4	0.0520	0.0420	0.3400	0.0920	0.3930	0.0980

Disclosure statement

The authors report no declarations of interest.

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