Inorganica Chimica Acta, *168* (1990) 283–288

Chelating Agents as Antitumour Drugs: Formation of Chelating Agents from the Antitumour Pro-drug, Razoxane

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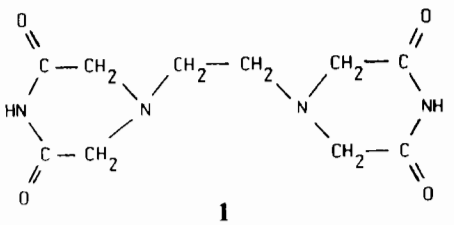
(Received July 26, 1989)

Abstract

The hydrolysis of the antitumour drug Razoxane, i.e. 1,2-bis(3,5-dioxopiperazin-1-yl)propane, in the presence of copper(II) has been studied by pH stat methods at 25 °C, in the pH range 4–5, at metal ion concentrations 0.018–0.053 M, and ionic strength 0.3 M. In all cases the concentration of Razoxane was 5.7×10^{-3} M and the solvent system used was DMF/H_2O (1:2$\frac{1}{2}$). Under these conditions the reaction rate was found to be first order in Razoxane, hydroxide ion and metal ion concentrations indicating that base hydrolysis of an incompletely formed copper(II)–Razoxane complex was being observed. At copper(II) concentrations of 0.018 , 0.036 and 0.053 M the second order rate constants for base hydrolysis are 1.1×10^8, 2.3×10^8 and 4.0×10^8 M^{-1} min^{-1} respectively. The last of these is 2.2×10^8 faster than the second order rate constant for hydrolysis of the free ligand for which k_2 is approximately 1.8 M^{-1} min^{-1} at 25 °C, ionic strength 0.3 M. Since there is only limited formation of the copper(II)–Razoxane complex the rate enhancement due to the metal ion is vastly in excess of 2.2×10^8. The reaction mechanism involves nucleophilic attack by a hydroxy ligand on the juxtaposed carbonyl group to give a strained intermediate which then undergoes rapid decomposition. Only one of the imide rings in Razoxane is hydrolysed in this reaction. Nevertheless a strong chelating agent is produced. The mode of action of Razoxane as an antitumour drug may involve hydrolytic metabolism to such a chelating agent.

Introduction

Many drugs which retard the growth of tumours are chelating agents and are thought to act by interfering with metalloenzymes which are necessary for the rapid growth of malignant cells [1, 2]. Some other anticancer drugs, although not chelating agents themselves, are metabolised to chelating agents *in vivo* [1, 2]. One of the most powerful chelating agents, EDTA shows no antitumour activity and this is because of its polarity which prohibits it from penetrating intracellular sites. In the early 1970s a range of non-polar EDTA derivatives was synthesised at the Imperial Cancer Research Fund Laboratories, London and these were tested for antitumour activity [1]. It was thought that the non-polarity of these reagents would allow them to cross cell membranes after which they could be hydrolysed intracellularly to active, chelating metabolites. Nonetheless the tetramethyl and tetraethyl esters both proved inactive and this probably because of their hydrolytic reactivity, causing them to be hydrolysed extracellularly [3]. The diimide of EDTA,1,2-bis(3,5-dioxopiperazin-1-yl)ethane (1)



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gave encouraging results in initial screening tests and this pointed the way to an investigation of compounds containing a systematic variation of structure within the diimide framework. Activity was found to be retained by methyl but not by ethyl substitution on the central ethylene chain

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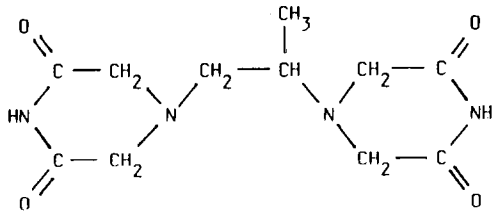

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and the enantiomers and racemate of 1,2-bis(3,5-dioxopiperazin-1-yl)propane (**2**) were found to be particularly active as antitumour agents [4, 5]. This compound to which the official name Razoxane has been given by the British Pharmacopoeia Commission [5], is mainly used in combination with radiotherapy to treat all forms of soft tissue-, chondro- and osteosarcomas [6]. It is also used alone or in combination in the treatment of malignant lymphomas and acute leukaemias [6].

The cytotoxicity, cell cycle specificity, anti-metastatic activity, pharmacology, toxicology and clinical testing of this drug have been reviewed [4, 5]. It has been shown that Razoxane is hydrolysed intracellularly and two unidentified metabolites have been detected, neither of which is the tetra-acid [7]. We have investigated the hydrolysis of Razoxane *in vitro* in the presence of metal ions which promote the reaction. In the presence of copper(II) the hydrolysis product appears to be one in which only one of the imide rings is open. In this paper the kinetics and mechanism of this hydrolysis is described.

Experimental

Razoxane was synthesised by heating propane-1,2-diaminotetraacetic acid (0.15 mol) and formamide (3 mol) under reduced pressure (200 mm) at 100–120 °C, for 5 h [8]. Cooling the solution to room temperature afforded the product in 77% yield.

The hydrolysis of Razoxane in the presence of copper(II) was studied by the pH stat method using a Radiometer pH Meter 26 linked to a Titrator 11 and an Autoburette ABU 12 [9]. The addition of titrant (0.1 M NaOH) was monitored on a Titrigraph REA 160. The reaction solutions contained Razoxane (5.7×10^{-3} M) and copper(II) nitrate (0.018–0.053 M) dissolved in DMF/H_2O (35 cm^3, 1:2.5), with an appropriate quantity of $NaClO_4$ added in order to maintain ionic strength constant at 0.3 M. Values of k_{obs}, the pseudo first order rate constant for the reaction, (see eqn. (2) in 'Discussion') were obtained either from plots of $\ln(V_\infty - V_t)$ versus t where V_∞ is the final volume of titrant added and V_t is the volume at time t, or from Guggenheim plots [10] of $\ln[V_{t+2t(\frac{1}{2})} - V_t]$ versus t where $t(\frac{1}{2})$ is the half life of the reaction. Good linear plots were obtained by both methods of analysis. Solutions of copper(II) nitrate were standardised using EDTA [11]. An estimate of the rate constant for base hydrolysis of Razoxane itself was obtained also by the pH stat method at 25 °C and at pH 12.00. The solvent system used was DMSO/H_2O ($1:2\frac{1}{2}$) and the solution was 5.7×10^{-3} M in Razoxane with sufficient $NaClO_4$ added to give an ionic strength of 0.3 M.

Results and Discussion

Razoxane is an antitumour drug which was originally designed on the basis that its action would depend on it being metabolised intracellularly possibly by enzymatic hydrolysis to a chelating agent which would interfere with metalloenzymes necessary for the growth of tumour cells [1]. There are three possible direct hydrolysis products of Razoxane (discounting subsequent amide hydrolysis reactions) under physiological conditions, as shown in Scheme 1. In one of these (**5**) both imide rings are opened and the reaction involves the release of $2H^+$ per molecule of reactant. The other two products, **3** and **4**, result from opening of one ring and only $1H^+$ per molecule is released during their formation. All three products are potential chelating agents and any one could be the antitumour active metabolite.

Under normal pH and temperature conditions Razoxane undergoes slow hydrolysis and we therefore decided to investigate the catalytic effects of metal ions such as copper(II) on this reaction. In the presence of copper(II), complex formation may be represented by Scheme 2. At pH 4–5, T = 25 °C, I = 0.3 M, a hydrolysis reaction was observed as indicated by acid release. The reaction was studied by the pH stat method at pH 4.02, 4.51 and 5.01 and at $[Cu^{2+}]$ = 0.018–0.053 M. At each pH and at each $[Cu^{2+}]$ studied the reaction followed first order kinetics and the values of the first order rate constants are summarised in Table 1. These values are proportional to OH^- and Cu(II) concentrations leading to the rate expression for the reaction

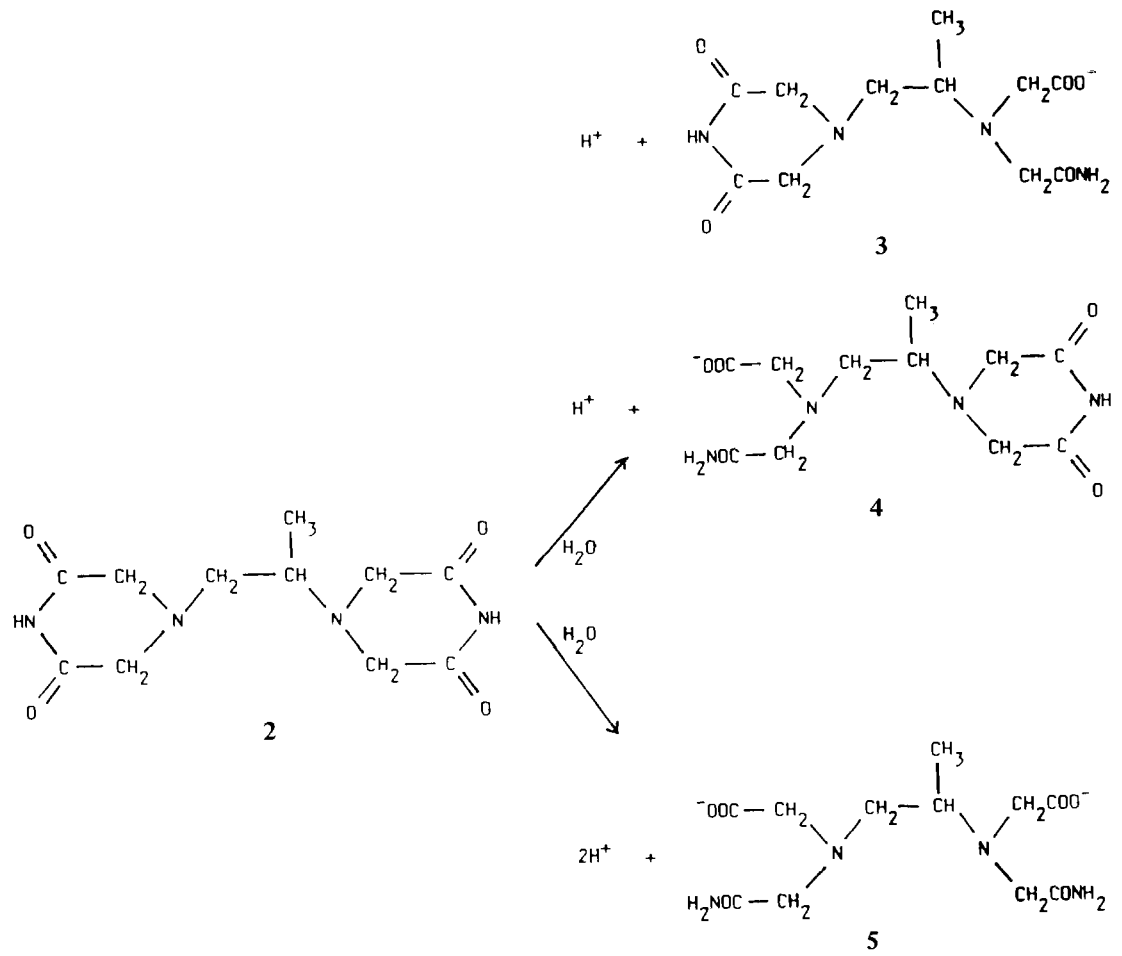
$$\text{Rate} = k_3[\text{Razoxane}][Cu^{2+}][OH^-] \quad (1)$$

At constant pH and [Cu(II)] this reduces to the first order expression

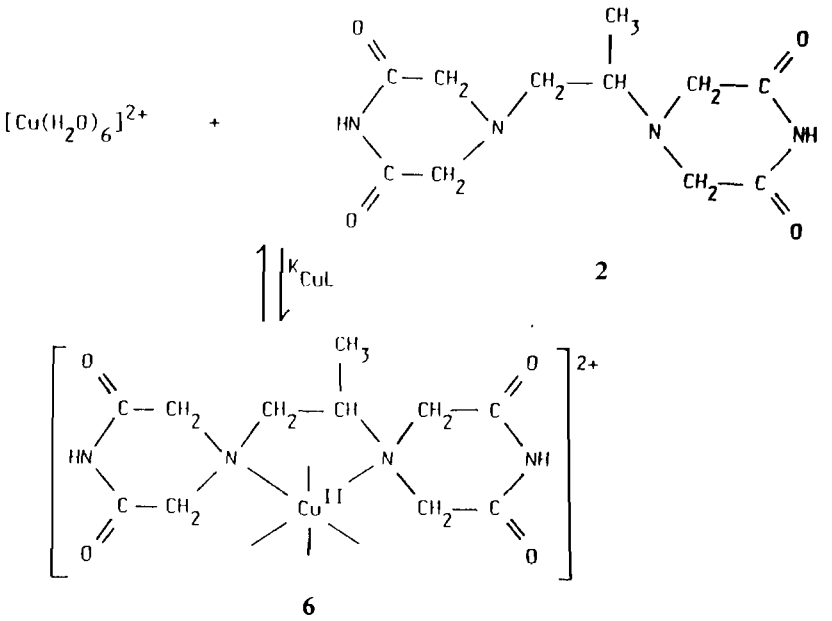
$$\text{Rate} = k_{obs}[\text{Razoxane}] \quad (2)$$

where $k_{obs} = k_3[Cu^{2+}][OH^-]$.

The first order dependence of the rate on the metal ion concentration is due to limited formation of the Cu(II)–Razoxane complex (**6**) [12]. The first order dependence of the rate on the hydroxide ion concentration implies that base hydrolysis of the



Scheme 1.



Scheme 2.

TABLE 1. Rate constants for the copper(II)-promoted hydrolysis of Razoxane at 25 °C, I = 0.3 M, showing the first order dependence of rate on (a) [OH^-] and (b) [Cu(II)]

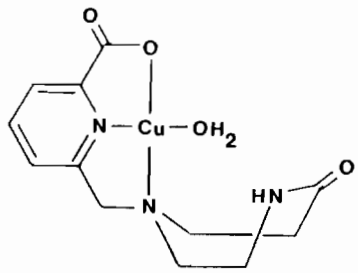
pH	k_{obs} (min^{-1})	$k_{obs}/[OH^-]$ ($M^{-1}\,min^{-1}$)
(a) [Cu(II)] = 0.018 M		
4.02	0.015	1.0×10^8
4.51	0.052	1.1×10^8
5.01	0.175	1.2×10^8
[Cu(II)] = 0.036 M		
4.02	0.036	2.4×10^8
4.51	0.096	2.1×10^8
5.01	0.349	2.4×10^8
[Cu(II)] = 0.053 M		
4.02	0.059	4.0×10^8
4.51	0.185	4.0×10^8
[Cu(II)] (M)	k_{obs} (min^{-1})	$k_{obs}/[Cu(II)]$ ($M^{-1}\,min^{-1}$)
(b) pH 4.02		
0.018	0.015	0.83
0.036	0.036	1.0
0.053	0.059	1.1
pH 4.51		
0.018	0.052	2.9
0.036	0.096	2.7
0.053	0.185	3.5
pH 5.01		
0.018	0.175	9.7
0.036	0.349	9.7

imide is being observed. The fact that this reaction has a half life of 2–45 min depending on the copper(II) concentration in the pH range 4–5 implies that the rate of hydrolysis is considerably enhanced by the metal ion. The base hydrolysis of the free ligand is very slow. At pH 12.0 and 25 °C the rate constant for its hydrolysis is 2.5×10^{-2} min^{-1}. This gives a second order rate constant for base hydrolysis of 1.8 M^{-1} min^{-1}. In the presence of 0.18 M copper(II), the second order rate constant for base hydrolysis is 1.1×10^8 M^{-1} min^{-1} (Table 1) giving a rate acceleration of 0.6×10^8. At [Cu(II)] = 0.036 M the acceleration is 1.3×10^8 and at [Cu(II)] = 0.053 M it is 2.2×10^8. Since the concentration of the copper(II)–Razoxane complex (**6**) is far from saturation the rate enhancement provided by this species can be considered to be far in excess of 2.2×10^8.

Although the metal ion promoted base hydrolysis of imides has not previously been investigated the kinetics and mechanisms of analogous reactions of amides have been well documented and the principles involved can be applied to the case under investigation. Metal ion catalysis of amide hydrolysis has recently been the subject of a detailed analysis [13]. The hydrolysis of amides at neutral pH is known to proceed with rate limiting breakdown of the tetrahedral intermediate, and the catalytic role of a metal ion must involve facilitation of the C–N bond cleavage step. This can result from the ability of metal bound water to serve as a general acid catalyst in protonating the leaving nitrogen or from the ability of the metal to facilitate the breakdown of the tetrahedral intermediate directly. If the breakdown of the tetrahedral intermediate is accelerated sufficiently the formation of this intermediate may become the rate determining step of the reaction and an additional catalytic effect of the metal on the formation of this intermediate may be realised. The catalytic effect of the metal ion on the formation of the tetrahedral intermediate may occur either by a carbonyl activation mechanism or by an intramolecular hydroxy ligand attack mechanism. Hence the base hydrolysis of the O-bonded DMF complexes $[M(NH_3)_5DMF]^{3+}$, M = Co(III), Rh(III), Ir(III), for which rate accelerations of $>10^4$ have been reported occur by mechanisms involving external OH^- ion attack on the activated amide carbonyl group [14, 15]. On the other hand the base hydrolysis of the complex

$$[Co(en)_2(NH_2CH_2C(=O)NH_2)OH]^{2+}$$

is $>10^7$ faster than for free glycinamide and occurs by intramolecular attack by coordinated OH^- on the amide group [16]. A rate enhancement of 1.6×10^6 is observed for the base hydrolysis of the Cu(II)–lactam complex (**7**) relative to the free lactam [17].



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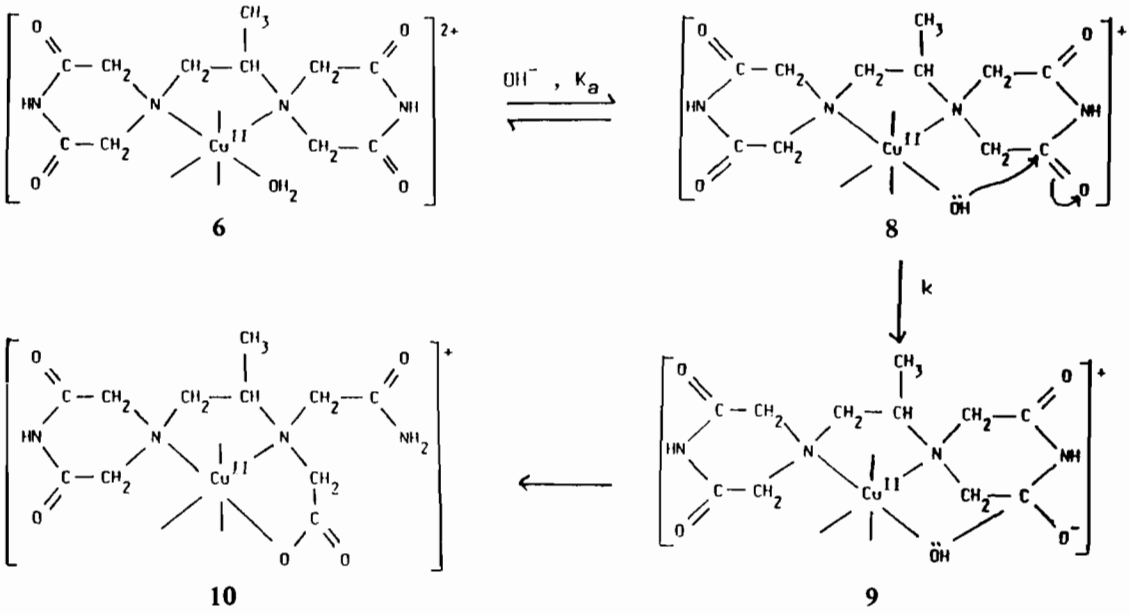
Since the carbonyl group is unlikely to be coordinated to the metal ion in this complex an intramolecular attack by coordinated hydroxide on the amide group is the more likely mechanism for hydrolysis.

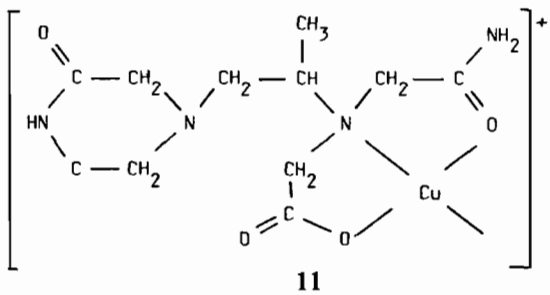
We propose an intramolecular hydroxide mechanism also for the base hydrolysis of the copper(II)–Razoxane complex, Scheme 3. In the copper(II)–Razoxane complex (**6**) the metal is most likely complexed to the two central nitrogen atoms. Be-



Scheme 3.

cause of steric constraints, verified using CPK molecular models, the carbonyl groups cannot also complex to the metal ion and hence are too far removed to be influenced by its electron withdrawing effect. Therefore the mechanism which involves external OH^- ion attack on an activated carbonyl group is not feasible and the alternative mechanism involving intramolecular attack by an OH^- ligand (conjugate base of a H_2O ligand) on the carbonyl group as shown in Scheme 3 is the favoured one. Because of its cyclic structure, the intermediate **9** containing the tetrahedral carbon is highly strained and its rate of decomposition will be accelerated to such an extent that it is likely that the rate determining step of the overall reaction is the formation of this intermediate. In this case the catalytic effect of the metal ion is twofold. It provides the hydroxide ligand for the intramolecular formation of intermediate **9** and it causes strain in this intermediate thus accelerating cleavage of the C–N bond.

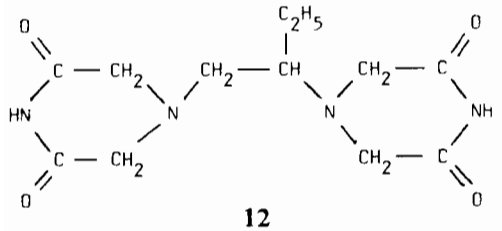
In all our experiments only one mole of hydroxide ion was consumed per mole of Razoxane. This implies that only one of the imide rings is undergoing hydrolysis. There are two possible explanations for this behaviour. Hydrolysis of one ring leads to a negatively charged ligand and produces a copper(II) complex (**10**) which is unipositively charged. The pK_a of an aquo ligand in this complex would be considerably higher than that of the aquo ligand in complex **6** and the concentrations of hydroxy complex would be too low in the pH range under investigation for hydrolysis to be observed (raising the pH caused precipitation of copper, which in all our experiments was present in excess). An alternative explanation lies in the fact that hydrolysis of one ring generates two new complexing sites – the carboxylate oxygen and the amide carbonyl group. As a result of the reaction therefore it is possible that the site of coordination of the metal ion changes from the central nitrogen atoms to a postion where it is complexed to only one of these nitrogens, and to the two oxygen atoms (**11**). This



would remove the second imide ring away from the influence of the metal ion. Although the reactions in Scheme 3 show the imide ring on the methyl substituent side of the ligand undergoing hydrolysis it is not possible to state on the basis of the available information whether it is this or the other imide ring which is labile. Further studies involving the isolation and crystallisation of the product complex and its structure determination by X-ray crystallography will resolve this.

The fully hydrolysed product of Razoxane (**5**) had previously been synthesised and the formation constants of its complexes with various metal ions studied [18]. This ligand binds particularly strongly

to Cu(II), Fe(II), Mn(II) and Zn(II) and the depletion of the intracellular levels of these ions as a result of the administration (and subsequent hydrolysis) of Razoxane may account for the effect of this drug in dramatically slowing down cell-cycle kinetics [18]. The possible inhibition of metalloenzymes such as superoxide dismutase by the hydrolysis product would be detrimental to the cells' defence against free radicals and may account for the efficiency of razoxane as an antitumour drug when used in combination with free radical producers such as radiation or certain quinones. The fact that the hydrolysis product of the antitumour inactive di-



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imide (**12**) shows similar metal binding properties as **5** except for an increased affinity for Zn(II) however is difficult to explain if the anticancer activity of Razoxane is based on chelation by its metabolite. It is therefore important to isolate the partly hydrolysed Razoxane product to establish whether it exhibits any discriminatory effects in complex formation reactions.

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