PHOTODYNAMIC EFFECTS IN THE COMPONENTS OF COTTON SEEDS

A. A. Tyshchenko, S. D. Gusakova,
I. P. Nazarova, and A. I. Glushenkova

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The formation of a stable radical cation and of an unstable peroxide form of gossypol in the light under aerobic conditions has been established on the basis of an analysis of the ESR spectra obtained. The formation of mono- and dianions of gossypol in its alkaline solutions has been shown.

Many reactions taking place in biological systemsin the presence of sensitizer substances, light, and oxygen include the transfer of excitation in a contact complex from a sensitizer to molecular oxygen O_2 with the formation of the excited singlet state $^1O_2$ and with the simultaneous quenching of the sensitizer. The transfer of an electron e^- with the formation of the oxygen radical anion O_2^- is a less probable event in the photodynamic effect [1]. A specific feature of the electron donor-acceptor (EDA) complexes arising in the latter case is shown in their dissociation into radical ions under the action of low-intensity light fluxes [2]. The electron spin resonance (ESR) method permits the radical cations or sensitizers formed in this process to be recorded [3] and in some cases it enables their structure to be determined [4].

It has been found by the ESR method that the illumination of the seeds of the number of plants with white light induces the formation in their coats of free radicals the concentration of which depends on the variety and amount of pigments, the moisture content of the seeds, and the intensity of the light [5]. The recording of a single ESR signal in gossypol — the main pigment of cotton seeds — in its alkaline alcoholic solution and the disappearance of the signal on contact of the solution with air have been reported [6]. When an alcoholic solution of caustic potash is poured into an alcoholic solution of gossypol an ESR signal consisting of four lines appears instantaneously [7]. We have found no other information in the literature available to us on the behavior of the ESR signal of gossypol with a change in the external conditions, including illumination, and on the products of the transformation of this pigment under the action of light and air.

In the present paper we give the results of a study of the photodynamic effects in cotton seeds and of the properties of their components that can cause these defects.

In whole seeds of a cotton plant of the variety Tashkent-1 we recorded an ESR signal similar to that described in the literature [5, 7]. Illumination of the seeds with light from an ordinary incandescent lamp led to an irreversible increase in the intensity of this signal by 150-200%, depending on the illumination dose. In the seed coat separated from the kernel the effect of illumination was retained, while in the unilluminated kernel the signal was practically absent but it appeared after illumination. The intensity of the ESR signal in the illuminated kernel was approximately two orders of magnitude lower than in the coat.

In the seed coat of a gossypol-less mutant of the cotton plant similar effects were observed with a somewhat smaller degree of increase in the intensity of the ESR signal but no signal was recorded in the kernel even on illumination. This permits the assumption that the presence of gossypol may be one of the reasons for the appearance of the ESR signal in irradiated cotton seeds. By irradiating a sample of (±)-gossypol under aerobic conditions, we found that both the intensity and the form of the ESR signal depended on the intensity of the incident light and the temperature. In the spectrum recorded under these conditions (Fig. 1, curve 1), an ESR signal with a width between the points of maximum slope $\Delta H = 15$ Oe is superposed on a narrow central signal with $\Delta H = 5$ Oe. The considerable asymmetry in the combined spectrum shows a displacement of the centers of the superposed signals relative to one another.

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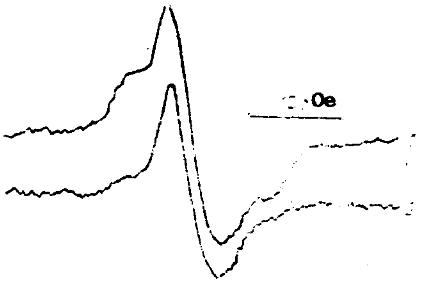


Fig. 1

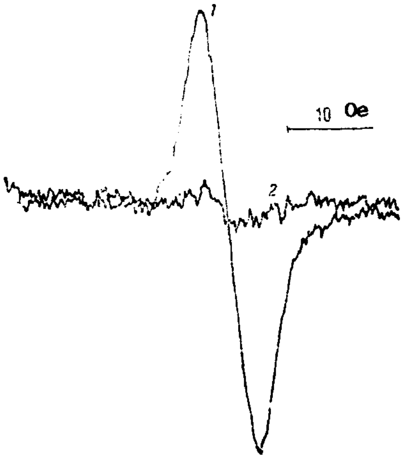


Fig. 2

Fig. 1. ESR spectrum of (±)-gossypol under aerobic conditions: 1) in the light; 2) after being kept in the dark for 40 h.

Fig. 2. ESR spectra of a solution of (±)-gossypol in CCl_4: 1) in the light; 2) in the dark.

The position of the center of the narrow signal corresponds to a g-factor of 2.002. The center of the superposed broad signal is shifted relative to the center of the narrow signal by 0.5 Oe in the high-field direction, which may show the presence of two different paramagnetic particles (PCs) in the sample of gossypol.

An experiment with saturation by the power of the microwave field [3] performed with a sample of illuminated gossypol showed that the narrow signal was saturated at an applied power of 20 mW, while the broad signal showed no signs of saturation even at 100 mW. The intensities of both signals rose with an increase in illumination regulated by a change in the distance from the source of light to the sample. A rise in the temperature of the sample from 20 to 90°C led to a fall in the intensities of both signals by 50%. After a previously illuminated sample of gossypol had been kept in the dark for 40 h at 20°C, the intensity of the broad signal had fallen by 80% (Fig. 1, curve 2), while the intensity of the central narrow signal had fallen by only 10% and it remained practically at this level for an indefinitely long time. After being kept in the dark for five days, the intensity of the broad signal fell to the level of the apparatus noise.

The combination of the above-mentioned facts — the difference in the g-factors of the broad and narrow signals and the different behaviors of the intensities after being kept in the dark and on saturation by microwave power — show that the signals observed do actually belong to two different PCs that are products of the photoreaction of gossypol with O_2. The stable type of PCs with the narrow signal must be assigned to the radical cationic form of gossypol $\Gamma^{+\bullet}$ formed on the photodissociation of its EDA complex with the isolation of the radical anion $O_2^{-\bullet}$. The less stable type of PCs with the broad signal obviously relates to an unstable peroxide radical $\Gamma{-}O{-}O^{\bullet}$ which is then converted into a diamagnetic molecule of gossypol peroxide or a hydroxy derivative of gossypol.

In view of what has been said, there is no point in estimating the number of PCs in gossypol from the intensity of its ESR spectrum [6], unless information is available on the prehistory of the sample: the intensity of the light falling on it, the size of the illuminated surface, and the temperature.

The dissolution of previously illuminated (±)-gossypol in CCl_4, CH_2Cl_2, CH_3OH, C_2H_5OH and in pure cottonseed oil triacylglycerols led to the disappearance of the ESR signal. The illumination of nonalcoholic solutions of gossypol caused the appearance of it of only the signal of the radical cationic form $\Gamma^{+\bullet}$ with $\Delta H = 6$ Oe (Fig. 2), clearly observable in the light over a wide range of temperatures and concentrations of the pigment but disappearing in solution in the course of a few minutes after the switching off of the light.

The disappearance of the ESR signal is undoubtedly due to exchange processes in the solutions as a result of which the stabilization of the radical cation form is limited to a far

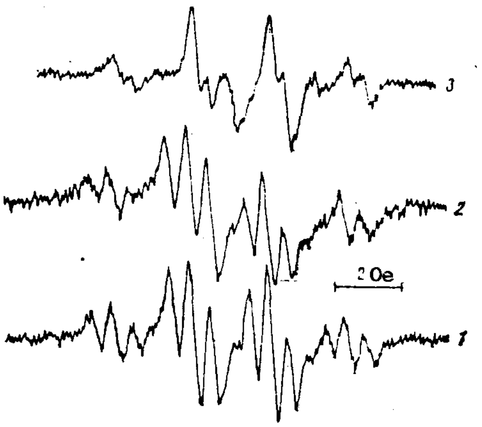
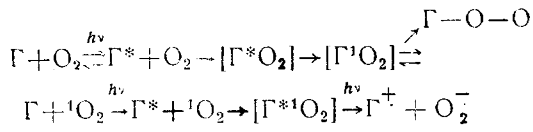


Fig. 3. ESR spectra of gossypol solutions: 1) in 0.1 N NaOH in H_2O under an aerobic conditions; 2) the same solution with access of air; 3) in 0.1 N NaOD in D_2O.

smaller time interval than is the case in dry gossypol. The absence of the broad ESR signal of the peroxide form of gossypol in solutions in the light may be considered as a consequence of the short lifetime of organic peroxide radicals in solutions [3]. It is obvious that only on the illuminated surface of dry gossypol do the conditions exist for the stabilization of its peroxide radicals which permit their signal to be recorded on illumination and several hours after the sample has been kept in the dark.

In alcoholic solutions of gossypol where illumination did not cause the appearance of any ESR signal whatever, obviously, the conditions for the stabilization even of the radical cation form of gossypol are absent. Here we must also take into account the fact that the lifetime of $^1O_2$ in alcohols is one of the shortest for condensed media and differs from that in chlorinated organic solvents and lipids by a factor of several hundreds [8].

What has been said above permits the following probable scheme of the behavior of gossypol in the presence of oxygen in the light to be suggested:



$$\begin{array}{r} \nearrow \Gamma - O - O^{\cdot} \\ \Gamma + O_2 \overset{h\nu}{\rightleftarrows} \Gamma^* + O_2 \rightarrow [\Gamma^* O_2] \rightarrow [\Gamma^1 O_2] \rightleftarrows \\ \Gamma + {}^1O_2 \xrightarrow{h\nu} \Gamma^* + {}^1O_2 \rightarrow [\Gamma^{*1}O_2] \xrightarrow{h\nu} \Gamma^{\dot{+}} + O_2^{\dot{-}} \end{array}$$

The fastest stage in the suggested scheme is the transfer of excitation from the carbonyl groups of gossypol Γ^* to O_2 in the contact complex $[\Gamma^*O_2] \rightarrow [\Gamma^1O_2]$. Because of the short lifetime of $^1O_2$, the subsequent reactions have a smaller probability. This is in harmony with the fact reported in the literature of the low yield of $O_2^{-\cdot}$ in reactions of sensitizers in the excited state [1]. Furthermore, the rapid breakdown of the EDA complex $[\Gamma^{*1}O_2]$ in the light that we have established by the ESR method gives grounds for excluding it from the direct precursors of the peroxide form of gossypol.

It remains to be assumed that the most probable precursor of the peroxide form with the contact complex of gossypol in the ground state with singlet oxygen is $[\Gamma^1O_2]$. It follows from this that in systems generating $^1O_2$ under dark conditions gossypol in high concentrations may exhibit antioxidant properties by forming the contact complex $[\Gamma^1O_2]$. In addition to this, the combination of a medium with a long lifetime of $^1O_2$ and a low concentration of Γ^* may lead to the appearance of its preoxidant properties through the generation of $^1O_2$ end of $O_2^{-\cdot}$ in the light. The latter must be borne in mind when kinetic measurements in systems containing gossypol are performed under conditions of illumination [9].

The results obtained in a number of investigations [9-11] permit the assumption that the physiological action of gossypol may be connected with the appearance of active forms of oxygen. It must be mentioned that normal energy metabolism in the cell requires alkaline pH values at which gossypol, like other polyphenols, is converted into the anionic form, which must be reflected on its donor-acceptor properties. In the light of this hypothesis, we have studied the behavior of gossypol under alkaline conditions by the ESR method.

The addition of gossypol to a 0.1 N aqueous solution of NaOH under the conditions of limited contact with air is accompanied by the appearance of an ESR signal with a hyperfine structure (HFS) (Fig. 3, curve 1), the form of which changes as air diffuses into the solution

(Fig. 3, curve 2), while the intensity of the signal is practically independent of the illumination of the solution. When the area of contact of air with this solution is brought to a minimum by enclosing it in a plane quartz cell with a distance between the walls of 0.1 mm, a gradual change in the color of the top part of the solution from yellow-green to cherry-red/brown is observed due to the slow diffusion of the products of the interaction of gossypol with O_2 from the surface into the bulk of the sample. The spreading of the cherry-red/brown coloration through the whole volume of the cell is accompanied by a broadening of the HFS lines as far as the complete disappearance of the spectrum.

The observed phenomena are connected with the transfer of e^- from the anionic form of gossypol that is formed under alkaline conditions to a O_2 molecule. At comparable concentrations of gossypol anions and O_2, the exchange of electrons between them leads to a broadening of the lines in the spectrum, while when the O_2 is present in excess, it leads to the disappearance of the spectrum and to the appearance of a new isolated assymmetric signal in almost the center of the spectrum that has disappeared. As can be seen from the results obtained, the transfer of e^- in the system takes place under dark conditions and does not depend on illumination.

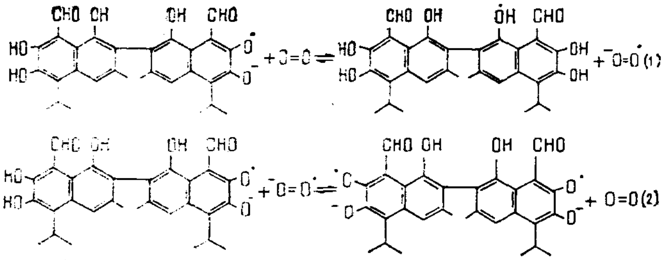
The spectrum presented in Fig. 3, curve 1, consists of a quartet of triplets with a binomial distribution of the intensities of the lines, 1:2:1 and 1:3:3:1, and with a g-factor of 2.002. This distribution of intensities is due to interactions of a lone electron in the anionic form of gossypol with two magnetically equivalent protons with a hyperfine splitting constant $a_{2H} = 0.6$ Oe and with three magnetically equivalent protons with $a_{3H} = 2.3$ Oe. The replacement of H_2O in the alkaline solution by heavy water, D_2O, permitted additional information to be obtained for the assignment of the constants observed.

Figure 3, curve 3 shows the ESR spectrum obtained on the addition of gossypol to a 0.1 N solution of NaOD in D_2O. The asymmetry observed in the center of the spectrum is due to the superposition of a signal appearing when O_2 is present in the solution. The retention in the spectrum of the splitting of the lines into a quartet with $a_{3H} = 2.3$ Oe (Fig. 3, curve 3) serves as a proof that it is caused by the interaction of an unpaired electron with three equivalent protons of one CH_3 group attached directly to the aromatic ring in the structure of gossypol. The conversion of the triplets of the lines of the quartets into doublets with a hyperfine splitting constant $a_{1H} = 0.6$ Oe is connected with the replacement of one of two magnetically equivalent protons responsible for the splitting into triplets by D.

The deuterium nucleus, with spin 1 must split the lines into triplets with an intensity ratio of 1:1:1 and with a somewhat smaller value of the constant than a_{2H} [3]. The small value of a_{2H} and the exchange of deuterium nuclei with the medium leads to the situation that the splitting due to the interaction of the unpaired electron with the deuterium nucleus expressed in a broadening of all the lines of the spectrum in D_2O (Fig. 3, curve 3) as compared with the spectrum in H_2O (Fig. 3, curves 1 and 2). Thus, one of the two magnetically equivalent protons responsible for the splitting of the lines of the quartet into triplets in the spectrum of the radical anion form of gossypol undergoes exchange with the medium.

The dissolution of gossypol in a concentrated aqueous solution of caustic soda caused the brief appearance of an intense ESR signal in the form of a doublet with splitting between the components of 2 Oe. The position of the center of the doublet was shifted by 0.2 Oe in the low-field direction as compared with the center of the spectrum of a 0.1 N alkaline solution of gossypol. The diffusion of air into the bulk of the solution led to the disappearnace of the doublet, obviously, because of the transfer of e^- to O_2. The replacement of H_2O by D_2O in this solution scarcely changed the form of the doublet. It was reported long ago that in a concentrated solution of caustic soda gossypol, losing carbonyl groups, is converted into apogossypol [12, 13]. It is obvious that the difference of the ESR spectrum obtained in this solution from the spectrum of gossypol in a 0.1 N solution of caustic soda is connected with just this circumstance.

In the initial gossypol, the spatial closeness and the presence of a hydrogen bond between the aldehyde and hydroxy groups may be responsible for the equality of the constants of hyperfine splitting on their protons, which gives grounds for considering these protons to be responsible for the splitting into triplets of the lines of the quartet in the spectrum of $\Gamma^{-\cdot}$ (Fig. 3, curve 1). In a concentrated solution of caustic soda the loss of the carbonyl groups by the gossypol and the redistribution of the electron density connected with it leads to the above-mentioned transformation of the ESR spectrum into an intense doublet. This relates to solutions not containing O_2. The contact of the solutions with air leads to the exchange of electrons with O_2 and to the formation of a dianion $\Gamma^{2\dot{-}}$.



Exchange interactions between unpaired electrons in the symmetrical parts of the dianion must, according to theory [14], cause a deviation in the distribution of the intensities from binomial, as is actually observed on the contact of a 0.1 N alkaline solution of gossypol with air (Fig. 3, curve 2). With an excess of O_2, the equilibrium (1) is shifted to the right, which leads to a disappearance of the ESR spectrum of alkaline solutions of gossypol when they are shaken in the air. Correspondingly, an excess of gossypol should lead to shifts of the equilibrium (1) to the left and of equilibrium (2) to the right, leading to the blockage of the reaction $O_2^{-\bullet}$ anions.

Alkaline alcoholic solutions of gossypol under anaerobic conditions possess ESR spectra with similar parameters, but in the presence of O_2 more extended spectra arise than in aqueous alkali. This fact can be evaluated as the influence of the medium on the interaction of O_2 with gossypol.

Thus, it may be considered that for the gossypol anion the form of the ESR spectrum (Fig. 3, curve 1) is due to the interaction of an unpaired electron with the protons of a terminal methyl group, with the proton of an aldehyde group, and with the mobile proton of a hydroxy group forming a hydrogen bond with an aldehyde group. Experiments with illumination of solutions of gossypol show that the transfer of an electron between the gossypol anion and oxygen may take place under dark conditions, while the transfer of an electron by gossypol and oxygen molecules requires their previous passage into excited states. These properties of gossypol may explain the photodynamic effects in cotton seeds and the tissues of the cotton plant.

EXPERIMENTAL

High-resolution spectra in the form of the first derivative of the absorption line were recorded on a Varian E-4 radiospectrometer with slow passage under conditions remote from the saturation of the signals. The samples were illuminated with light from an incandescent electric lamp with a power of 100 W placed in a OI-24 illuminator at distances from 0.3 to 3 m from the optical slit of the resonator. A layer of water 3 cm thick was used as a heat filter.

Seeds from the cotton plant of the Tashkent-1 variety obtained from ISSKh MSKh SSSR im. G. S. Zaitseva (G. S. Zaitsev Agricultural Institute, Ministry of Agriculture of the USSR) and the seeds of a gossypol-less mutant of the L-479 line kindly supplied by Sh. Turabekov of the Laboratory for Special Cotton-Plant Genetics (Tashkent State University).

(±)-Gossypol and the triacylglycerols were isolated from cottonseed oil by methods described previously [15, 16].

SUMMARY

1. In dry (±)-gossypol on its illumination under aerobic conditions, two different ESR signals were detected which have been assigned to the stable radical cationic and unstable peroxide forms of gossypol. In solutions of (±)-gossypol in chlorinated organic solvents only one ESR signal, from its radical cationic form, was recorded.

2. The formation of mono- and dianions in alkaline solutions of gossypol has been shown on the basis of an analysis of the hyperfine structure and changes in the ESR spectra on passing from anaerobic to aerobic conditions.

3. Gossypol may exhibit not only antioxidant but also prooxidant properties depending on the concentrations of gossypol in the medium and the lifetime of active forms of O_2 in it.

LITERATURE CITED

1. Kh. Fut, in: Free Radicals in Biology [in Russian], Moscow, Vol. 2 (1979), p. 96.
2. K. Yamaguchi, Y. Ikeda and T. Fueno, Tetrahedron, 41, 2099 (1985).

3. F. Gerson, High-Resolution in ESR Spectroscopy, Wiley, New York (1970).
4. A. A. Krasnovskii, A. V. Umrikhina, and N. V. Bublichenko, in: The Spectroscopy of Phototransformations in Molecules [in Russian], Leningrad (1977), p. 106.
5. S. A. Stanko, A. G. Chetverikov, G. P. Novikova, and V. Ya. Taran, Abstracts of the 1st All-Union Biophysical Congress [in Russian], Moscow, Vol. IV (1982), p. 48.
6. A. Sultanov, A. P. Ibragimov, Sh. Aripdzhanov, A. I. Ismailov, and A. S. Sadykov, Uzb. Biol. Zh., No. 2, 3 (1967).
7. A. P. Ibragimov, Radiation-Biochemical Effects in Cotton Seeds [in Russian], Fan, Tashkent (1969), p. 90.
8. M. N. Merzlyak and A. S. Sobolev, Itogi Nauki Tekh. Ser. Biofizika, No. 5, 118 (1975).
9. A. A. Aver'yanov and A. I. Ismailov, Nauchn. Dokl. Vyssh. Shkoly. Biol. Nauki, No. 5, 76 (1986).
10. A. De Peyster, A. Quintanilha, L. Packer, and M. T. Smith, Biochem. Biophys. Res. Commun., 118, No. 2, 573 (1984).
11. J. Reyes, J. Allen, N. Tanpaichitz, A. R. Bellve and D. J. Benos, J. Biol Chem., 259, No. 15, 9607 (1984).
12. R. Adams and D. J. Butterbaugh, J. Am. Chem. Soc., 60, No. 9, 2174 (1938).
13. I. P. Nazarova, A. I. Glushenkova, and A. U. Umarov, Khim. Prir. Soedin., 121 (1981).
14. V. N. Parmon, A. I. Kokorin, and G. M. Zhidamirov, Stable Biradicals [in Russian], Moscow (1980), p. 72.
15. I. P. Nazarova, G. A. Nezhinskaya, A. I. Glushenkova, and A. U. Umarov, Khim. Prir. Soedin., 608 (1979).
16. S. D. Gusakova, I. I. Vinokurov, and A. U. Umarov, Khim. Prir. Soedin., 288 (1981).

A NEW TERPENOID COUMARIN trans-DIVERSIN FROM *Ferula Litwinowiana*

S. V. Serkerov and N. F. Mir-Babaev

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The results are presented of investigations into the structure of a new terpenoid coumarin with the composition $C_{19}H_{20}O_4$ with mp 96-98°C (from aqueous ethanol), differing from diversion by the trans position of one bond. On the basis of spectroscopic (IR and ^1H NMR) results, the structure of 7-(3',7'-dimethyl-5'-oxoocta-3'E,6'-dienyloxy)coumarin is proposed for this substance.

Continuing a study of the chemical composition of the roots of *Ferula litwinowiana* K.-Pol. [1, 2], by chromatography on a column of alumina we have isolated a crystalline substance with the composition $C_{19}H_{20}O_4$, mp 96-98°C (aqueous ethanol). In the IR spectrum of the substance in the region of characteristic frequencies there are absorption bands at 1730 and 1715 cm^{-1} (CO groups of a δ-lactone and of a conjugated ketone) and at 1680, 1630, 1520 cm^{-1} (double bonds). A direct comparison of the IR spectra of diversin and the coumarin under investigation showed differences characteristic for isomeric compounds. A mixture of the samples being compared gave a depression of the melting point (it melted at 85-87°C).

The NMR spectrum of the substance isolated had the signals characteristic for a 7-substituted coumarin nucleus: doublets at 7.64 ppm (J = 10 Hz, H-4), 6.24 ppm (J = 10 Hz, H-3), and 7.38 ppm (J = 8.5 Hz, H-5), and a quartet at 6.86 ppm (J_1 = 8.5 Hz, J_2 = 2 Hz, H-6).

The H-8 signal, undergoing allyl interaction with H-6, was superposed on one component of the H-6 doublet and therefore appeared in the form of a broadened singlet at 6.80 ppm. In the NMR spectrum, three-proton singlets of vinylmethyl groups at 1.92, 2.20, and 2.26 ppm, two-proton singlets of vinylmethyl groups at 2.57 ppm (J = 7 Hz) and 4.21 ppm (J = 7 Hz) and one-proton broadened singlets of olefinic protons at 6.09 and 6.15 ppm were similar to the signals of the monoterpene moiety of the diversin molecule. However, the singlet of one

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V. L. Komarov Institute of Botany, Academy of Sciences of the Azerbaidzhan SSR, Baku.
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