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Fluorometric Detection of Nitric Oxide with Diaminofluoresceins (DAFs): Applications and Limitations for Plant NO Research

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Abstract Nitric oxide (NO), a reactive nitrogen species, serves as a signaling molecule in plants, animals, fungi, and bacteria. In spite of its potential significance, however, the unique challenges of NO research can bring confusion to investigations, primarily due to difficulties in detecting and quantifying biological NO production. To overcome such barriers, we recommend that researchers choose a combinatorial approach for monitoring NO levels, in which multiple methods having distinct detection principles are employed. After an overview of the major methodologies for NO detection, we highlight the usefulness and application limits of the fluorescence probe diaminofluorescein (DAF), which has been preferentially applied in studies of plant NO-producing systems.

1 Introduction

Nitric oxide (NO) is a ubiquitous molecule that exists in interstellar space, the atmosphere, and soils. As one of the nitrogen oxides (NO_x) produced through the combustion of fossil fuels it is also recognized as a harmful air pollutant (Yamasaki 2000). The discovery of NO synthase (NOS), which produces NO from O_2 and arginine, combined with our growing understanding of the pivotal roles for NO in living cells has offered a new paradigm to the fields of biochemistry, cell biology, and physiology: NO as an important signaling molecule not only in plants but also in animals, fungi, and bacteria (Yamasaki 2004).

Scientific breakthroughs, in many cases, have been sustained by developments of new methods or technologies; research in NO biology and biochemistry is no exception. Reliable methods for the specific detection and quantitative determination of NO production in tissues, cells, and organelles are prerequisite for a better understanding of the complex biological functions of the radical gas molecule. It is undeniable that development of new

NO detection methodologies has promoted recent advances in NO research in living systems. Biogenic NO can be detected either by direct means or in the form of its stable oxidized products nitrite (NO_2^-) and nitrate (NO_3^-). In this chapter, we describe NO detection technologies that have been applied to plant systems. After a brief review of these methods we focus on potential problems and common errors encountered in utilizing diaminofluorescein fluorescence technique for detection of NO.

2 Griess Assay

The assay developed in the 19th century by Johann Peter Griess to detect nitrite by the formation of a colored diazo compound in an acidified solution (Ivanov 2004) has been widely adopted for use in modern NO research. The most common refinement of the Griess assay, available in over a dozen commercially available kits (Sun et al. 2003), precedes via initial reaction of nitrite (or more precisely, its acidified unstable product N_2O_3) with sulfanilamide to form a transient diazonium salt followed by addition of *N*-naphthylethylenediamine to yield a stable azo compound that absorbs strongly at 540 nm. A nitrite standard reference curve should be prepared for each assay. The practical sensitivity limit for this procedure is approximately 0.5–3 μM nitrite, depending on the matrix.

For a more accurate measurement of NO produced in a sample one must also measure the nitrate formed via oxidation of nitrite. This is most often accomplished by reducing nitrate to nitrite immediately prior to introduction of the Griess reagents. Reduction of nitrate can be carried out by treating samples with purified nitrate reductase (NR) or appropriate reductants; the official method of the American Official Analytical Chemists calls for use of cadmium, a hazardous waste. Problems with reaction efficiency, interference, and labor demands of the nitrate-to-nitrite reduction step have been described by Sun et al. (2003). To those who have the available instrumentation, ion chromatography offers a cleaner, more convenient, and sensitive alternative for end-point nitrite/nitrate detection (Dionex 1996).

In plant systems, tissue sometimes contains a high amount of nitrate (or nitrite), a condition where a low level of NO production is difficult to determine due to the high background. This is the case in green leaves and roots where nitrate assimilation takes place (Kawamura et al. 1996). It is important to be reminded that the oxidation product, nitrite, is also a substrate of NO production (Yamasaki et al. 1999; Yamasaki 2000). This complexity in the relationship between nitrite and NO could potentially result in inaccurate quantification of biological NO production in plant systems (Yamasaki 2005). Furthermore, the procedure for the conversion of nitrate to nitrite using NR also includes a potential risk because the enzyme itself catalyzes

not only nitrate reduction but also NO production via nitrite reduction in a condition of excess reductants (Yamasaki and Sakihama 2000). Therefore, one should take these characteristics into consideration in quantitatively determining NO production activity in plant cells. Nevertheless, the Greiss assay is useful because it does not require specific instruments and skills; the assay is recommended for quantification of NO production in a sample that does not include a substantial amount of nitrate (or nitrite) or in a purified in vitro system.

3 Oxyhemoglobin

NO can bind to heme-proteins including hemoglobins and changes the absorption spectrum as a result. The binding of NO to oxyhemoglobin (oxyHb) can be detected photometrically in real-time as a spectral shift. Key early investigations of NO biology utilized this assay but subsequent findings have revealed several drawbacks, including interference by other compounds and NO_x that are commonly encountered in NO assays (Artz and Thatcher 1998; Privat et al. 1997; Schmidt et al. 1994). Conversely, actual NO production may be missed by this assay (Adak et al. 2002a,b). The need to purify commercially obtained oxyHb reagent (Taha 2003) is further reason to avoid this method. The obvious advantage and disadvantage of this method are similar to those of Griess method: no requirement for a specific instrument but low specificity. The method is applicable to an in vitro purified system.

4 Electron Spin Resonance

NO is a radical molecule that is paramagnetic, a characteristic that can be distinguished from other non-paramagnetic molecules. Electron spin resonance (ESR) or electron paramagnetic resonance (EPR) is applicable as another NO detection method that offers an advantage in real-time continuous monitoring of NO production. In addition, this method can be widely applied for NO detection in vivo because it can detect NO at room temperature. However, the ESR method involves two apparent difficulties: its low sensitivity and higher equipment cost (Taha 2003; Yao et al. 2004). The application of a spin trapping agent is an option to overcome the difficulty of low sensitivity. The organic radical PTIO has been applied as a spin trapping agent specific for NO (Akaike et al. 1993; Akaike and Maeda 1996). Membrane permeability of such a spin trapping agent should be considered when one applies the method to an in vivo system.

5
Electrochemical Sensors

The advantages and limitations of electrodes for detection of NO are well-summarized in a recent review by Taha (2003), who had a pivotal role in developing these technologies. Briefly, a chemically modified electrode surface is positioned behind an NO-selective permeable membrane, and oxidation of NO at the surface is detected as a change in amperage. Previously, electrodes could be applied strictly for liquid phase NO measurement but are now available for gas-phase measurement (Taha 2003).

Detection levels are as low as 0.3–0.5 nM and a linear range can be achieved at up to 25 μM NO (Mantione and Stefano 2004; Taha 2003; Zhang and Broderick 2000). Considering the short half life of NO in oxic environments, the thin 100 nm diameter of one available nanosensor is an especially desirable feature for reaching source cells (Zhang and Broderick 2000); a recent evaluation found this electrode to perform with markedly better reliability and durability than past probes (Mantione and Stefano 2004). Being electrochemical sensors, NO sensors are sensitive to temperature fluctuations but newer models are available with a temperature compensation option (Taha 2003). Experimental designs should control for the possibility of artifacts resulting from even low intensity illumination of a sample.

6
Chemiluminescence

In the chemiluminescence assay NO is reacted with ozone, producing excited-state NO_2, which upon decay to the ground state releases a photon that is detected by a photomultiplier. It is considered a useful method because of its high sensitivity and capability for real-time monitoring of NO but is limited to detection of gasiform NO (Taha 2003; Yao et al. 2004). End-point detection of NO produced in fluids requires reduction of nitrite and nitrate to NO by vanadium (III) chloride in hydrochloric acid at 90 °C. This method is ideal for monitoring NO release from plants (Rockel et al. 2002).

7
Fluorescence Methods

In an oxic environment N_2O_3, the immediate unstable product of NO oxidation, can react with various conjugated ring-containing compounds, such as diaminonaphthalene, dihydrorhodamine, or diaminofluorescein (DAF), resulting in the formation of fluorophores (just as N_2O_3 from nitrite acidification converts the Griess reagents to a stable chromophore). Near stochiometric real-time detection of NO is possible in by use of a fluorimeter for liquid samples or by fluorescent or confocal microscopy for cellular localization. Synthesis of new DAF derivatives in recent years has expanded the permissible pH range for detection and allowed for entry of the reagent into cells.

7.1
Application of DAF

Diaminofluorescein-2 (DAF-2) was the first product in the DAF series to become commercially available (Kojima et al. 1998). DAF-2 detects NO through the formation of a triazole molecule (DAF-2T) that exhibits strong green fluorescence. DAF-2DA, a diacetate analog of DAF-2, was designed in particular for imaging of NO produced in cells (Kojima et al. 1998). The diacetate form possesses an obvious advantage in efficient uptake of the fluorescent probe into the living cells; the intracellular DAF-2DA is quickly transformed to DAF-2 by endogenous esterase activities, enabling visualization of in vivo NO production. Because of high sensitivity, specificity, and simplicity, DAF-2DA has been extensively applied in many fields of NO research (Cohen and Yamasaki 2003; Lacza et al. 2003). The detection of NO with DAF fluorescence techniques is now the most widely used methodology in plant research.

Figure 1 shows an example for the measurement of NO production in *Chlamydomonas* cells with DAF-2DA (Sakihama et al. 2002). NO produc-

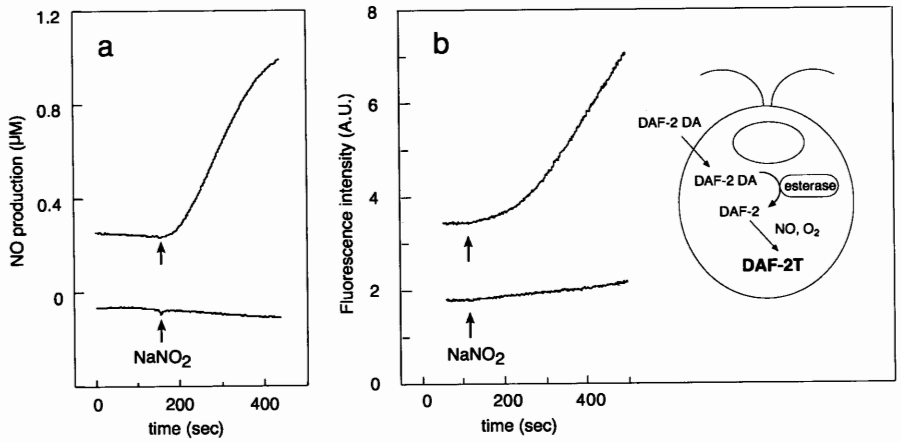

Fig. 1 Detection of extracellular and intracellular NO productions in the unicellular alga *Chlamydomonas reinhardtii*. **a** Time courses for NO production measured through NO released into an extracellular aqueous phase with an NO-specific electrode. **b** Time courses for NO production monitored with the intracellular NO indicator DAF-2DA. In both panels the *upper* and *lower traces* present NO production in the wild type and an NR-less mutant, respectively. Nitrite (5 mM sodium nitrite) was added to initiate NO production

tion in the bulk aqueous phase outside the cells was monitored with an NO electrode following addition of nitrite (Fig. 1a). Because of the membrane-permeable characteristic of DAF-2DA, one can monitor endogenous NO production within the cells in a real-time fashion. Figure 1b represent time courses for nitrite-dependent NO production within the *Chlamydomonas* cells using a fluorescence spectrophotometer. As in the NO electrode measurement, fluorescence intensity of the DAF by wild-type cells is increased in response to a nitrite supply. Because nitrate reductase (NR) is responsible for the NO production from nitrite, in both assay systems the NR-less mutant does not show any increase in the signals. Figure 2b shows visible fluorescence from DAF-2DA-incubated wild-type *Chlamydomonas* cells in the presence of nitrite. In good accordance with the time course measurements of endogenous and exogenous NO production, NR-less mutant cells do not show green fluorescence but exhibit red chlorophyll fluorescence only (Fig. 2c). When DAF-2DA is applied to a seedling of *Arabidopsis*, stomata of leaves and roots shine brightly (Fig. 2e,f), suggesting active NO production in these tissues.

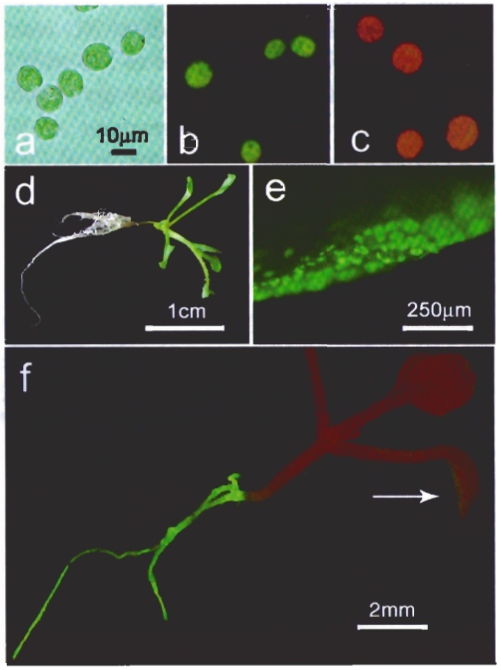

Fig. 2 Visualization of NO production with DAF-2DA. **a** *Chlamydomonas* cells (light microscope). **b** *Green* fluorescence from *Chlamydomonas* wild-type cells in the presence of nitrite. **c** *Red* chlorophyll fluorescence from *Chlamydomonas* NR-less cells in the presence of nitrite. **d** A seedling of *Arabidopsis thaliana*. **e** *Green* fluorescence from stomata of an abaxial side of a leaf. **f** Fluorescence image of a seedling of *A. thaliana*. The *arrow* indicates *green* fluorescence from stomata

7.2 Potential Errors in DAF Measurement

In addition to the development of NO detection methodology, there have been strong demands for NO scavengers. Until recently, only a few endogenous compounds such as hemoglobin and glutathione (GSH) were known to act as NO scavengers but with low specificity for NO (Herold 2003; Folkes and Wardman 2004). Development of 2-(4-carboxyphenyl)-4,5-dihydo-4,4,5,5-tetramethyl-1*H*-imidazolyl-1-oxy-3-oxide or cPTIO by Akaike and coworkers solved this problem. Now, cPTIO is widely used as an effective NO scavenger (and also as a spin trapping agent), owing to its high specificity and stoichiometric reaction with NO (Akaike and Maeda 1996). The combination of DAF and cPTIO has been frequently applied for proving in vivo NO production in animals (Pittner et al. 2003; Urno et al. 2005), bacteria (Creus et al. 2005), and plants (more than 20 papers as of 2006).

Figure 3 demonstrates the in vitro effect of cPTIO on fluorescence intensity of DAF-2T, which is formed from DAF-2. In the presence of cPTIO its fluorescence intensity is significantly increased. In principle, cPTIO acts as an NO scavenger through an oxidation reaction producing cPTI and NO_2. Contrary to the widely held presumption, the NO scavenger cPTIO does not suppress but actually enhances conversion of DAF into the DAF-2T fluorophore.

Figure 4a represents such a fluorescence enhancement effect of cPTIO on DAF fluorescence. In the presence of 0.1 mM cPTIO, visible green fluorescence is not inhibited but rather is increased. It should be noted that cPTIO itself has an intense color that poses another potential problem in

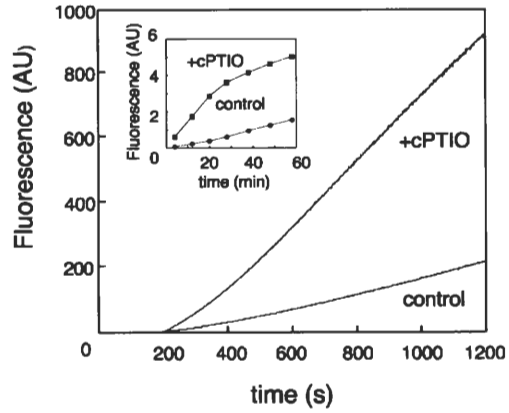

Fig. 3 Increase in DAF fluorescence in the presence of cPTIO monitored with a fluorescence spectrophotometer. The reaction medium contained 10 μM DAF-2, 250 μM SNAP, and 100 mM sodium phosphate (pH 7.4) with or without 100 μM cPTIO. *Inset* DAF fluorescence monitored with a microplate reader

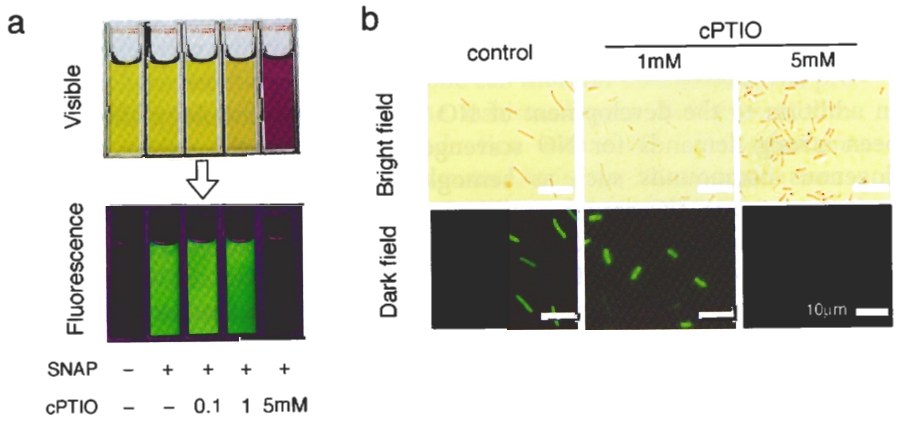

Fig. 4 Non-specific optical masking of fluorescence detection by cPTIO. **a** Different concentrations of cPTIO were added to each solution in quartz cuvettes. Black light was applied to the cuvettes for visual observation of DAF fluorescence. **b** Fluorescence microscopy images of *E. coli* cells expressing the green fluorescent protein in the presence or absence of cPTIO

fluorescence detection. The peak absorption wavelengths of cPTIO are 340 and 558 nm whereas the peak fluorescence emission of DAF-2T is 518 nm at 25 °C in 0.1 M phosphate buffer at pH 7.4. There is an obvious overlapping of the cPTIO absorption spectrum onto the DAF-2T fluorescence emission spectrum. Due to this, NO-dependent green fluorescence is not observed in the presence of a high concentration of cPTIO (Fig. 4a). To verify the masking effect of cPTIO on green fluorescence, Fig. 4b demonstrates the effect of cPTIO on green fluorescence from the green fluorescence protein (GFP) expressed in *Escherichia coli*. The excitation and emission spectrum of GFP is 475 nm and 509 nm, which is close to that of DAF-2T. It is important to be reminded that the principle of GFP fluorescence is irrelevant to NO production but its emission spectrum (emission max: 509 nm) is close to that of DAF-2T. Importantly, 5 mM cPTIO suppressed not only the fluorescence of a solution containing DAF but also that of GFP (Fig. 4b), suggesting that cPTIO could reduce NO-dependent DAF fluorescence due to the overlapping emission spectra. These results indicate that the application of cPTIO to the DAF system includes the potential risk of masking green fluorescence, irrespective of its origin.

7.3 Requirement for Oxidants in Fluorometric NO Detection by DAF

Kojima et al. have pointed out that DAF does not react with NO directly but with nitrous anhydride (N_2O_3) to form the fluorescent triazole compound (Kojima

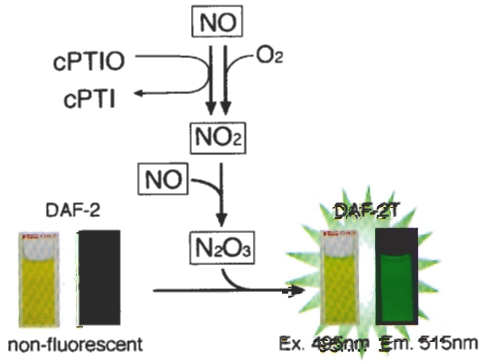

Fig. 5 Schematic diagram for the cPTIO reaction in fluorescence NO detection by DAF-2. NO is oxidized to NO_2 by either O_2 or cPTIO (without the requirement for O_2) and further changed to N_2O_3 via reaction with NO to form the highly fluorescent compound DAF-2T

et al. 1998). N_2O_3 is formed in the following scheme (Miles et al. 1996):

$$NO + O_2 \rightarrow 2NO_2 \quad (1)$$

$$NO_2 + NO \rightarrow N_2O_3\,, \quad (2)$$

where $k_1 = 2 \times 10^6\ M^{-1}\ sec^{-1}$ and $k_2 = 2 \times 10^9\ M^{-1}\ sec^{-1}$. Under ambient conditions, the oxidation proceeds through the reaction of NO with molecular oxygen (O_2) dissolved in solution (1). As presented in Fig. 5, cPTIO can eliminate NO to produce NO_2 and subsequently N_2O_3 in the reaction series (rate constant, $k = 1.01 \times 10^4\ M^{-1}\ sec^{-1}$) (Akaike et al. 1993; Akaike and Maeda 1996; Zhang and Hogg 2002). Faster formation of fluorescent DAF-2T rather than suppression could be observed in the presence of cPTIO owing to production of N_2O_3. Similar reactions with DAF have been reported in the case of PTIO, an analog of cPTIO. Therefore, cPTIO and PTIO do not suppress but enhance NO-dependent DAF fluorescence detected under ambient conditions.

In principle, the DAF–NO detecting system requires O_2 to form the fluorescent molecule, as mentioned above (Fig. 5). It should be noted that cPTIO can mimic the oxygen effect by acting as an oxidant for NO (Fig. 5). A saturated concentration of O_2 in water can be 0.253 mM at 25 °C but this is strongly affected by conditions. There is a wide range in O_2 tension among tissues and cell compartments: high in oxygen-evolving chloroplasts of plants and low in muscles of animals. The DAF-NO detecting system does not work in anoxic conditions, but supplementing cPTIO into anoxic compartments or cells may enable us to detect NO even in the absence of O_2. This aspect could provide an opposite application for cPTIO, namely, not to scavenge NO but to visualize the radical in anoxic compartments. For example, the detection of NO from cells in anoxic or hypoxic conditions could be another potential application of cPTIO for the DAF system.

8 Concluding Remarks

In a half-century, biochemistry and molecular biology have established many new technologies that enable us to investigate proteins (enzymes), lipids, sugars, and nucleic acids (DNA and RNA). NO has unique characteristics contrary to these conventional biomolecules: simple, small, ubiquitous, and unstable. Despite the potential significance of NO in plant physiological functions, however, our progress in this field is not satisfactory. Even the source of intracellular NO production has not yet been clarified, neither in plants nor in vertebrate animals. One apparent reason can be ascribed to the difficulty of detection and quantification of NO due to its uniqueness.

To date, two enzymes have been recognized for their capacity to produce NO in plants: nitrate reductase (NR) and NO synthase (NOS). Because mammalian NOS enzymes produce NO from O_2 and arginine, plant NO-producing activity is often estimated through measurement of citrulline product formation (citrulline assay). It should be noted that such an estimate rests on many assumptions. Often overlooked in studies of NO production by eukaryotes is the potential for bacterial associates, some of which are endophytes that cannot be removed by surface sterilization, to contribute NO via nitrification, denitrification, or NOS activity (Cohen et al. 2006; Creus et al. 2005; Cohen and Yamasaki 2003). In relying on production of citrulline from arginine as a measure of NOS activity one must keep in mind that this activity is shared by arginase, an enzyme not uncommon among bacteria (Xu and Verstraete 2001). Although a bacteria-free assay can be ensured by simple passage of an extract through a 0.2 μm filter, the possibility of an ultimate prokaryotic origin for any given NO-producing activity should always be taken into consideration. Definitive evidence of plant-encoded NO production should include transgene expression and demonstrated NO-producing activity of a cloned product along with subcellular localization of the enzyme in its native condition.

The DAF and DAF-DA series are powerful tools for the exploration of plant NO biology. There is no doubt that this fluorescence technique is one of the best options for NO detection due to its high sensitivity and simple procedure. In the application of this method to living systems, however, we must be aware of potential errors that can be avoided by knowing the reaction principle. It is always true that there is no perfect method in experimental science; awareness of application limits is needed to avoid misinterpretation of data. This is particularly true for the detection of NO in living systems. Therefore, we strongly recommend employment of at least two methods having distinct detection principles when initiating a study of an NO-producing activity in an in vivo system.

Acknowledgements Due to space limitations we were not able to cite many brilliant works on plant NO research that have applied the methods described in this chapter. Please refer to other chapters for such investigations. This work was supported by the grants (Grant-in-Aid for Scientific Research (B) and (C) to HY, and the 21st Century COE program of the University of the Ryukyus) from the Japanese Ministry of Education, Science, Culture and Sports.

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