Based on the Cyanine Derivatives’ Synthesis and Characterization of Ratiometric Fluorescent Probes to Detect pH

Xuhang Wang1,*

1HUANG POWER INTERNATIONAL INC. YINGKOU POWER PLANT
YingKou, LiaoNing, 115007

*Corresponding author e-mail: 352141300@hpiic.org

Abstract: Cyanine derivatives, the raw materials of classic near-infrared fluorescence probes, have been often used and developed to study imaging under different spectroscopes. In this paper, 4-carboxyphenylhydrazine hydrochloride, methyl iodide and p-hydroxybenzaldehyde were used as starting materials. After three-step reaction, compound (E)-5-carboxy-2-(4-hydroxy-styrene)-1, 3, 3-Trimethyl-3H-indole iodide (Compound 1). The intermediate and final products have been characterized by hydrogen spectroscopy, carbon spectroscopy and high-resolution mass spectrometry. This article mainly includes the following two parts: 1. Synthesis of (E)-5-carboxy-2-(4-hydroxy-styrene)-1, 3, 3-trimethyl-3H-indole iodide 2. The characterization part of the final product and the intermediate product is mainly hydrogen spectrum, carbon spectrum and mass spectrum.

Keywords: (E)-5-carboxy-2-(4-hydroxy-styrene)-1, 3, 3-trimethyl-3H-indole iodide; pH ratio fluorescent probe; 5-carboxy-1, 2, 3, 3-Tetramethyl-3H-indole iodide

1. Introduction

1.1 Research on fluorescent probes

1.1.1 The emission principle of fluorescence
Fluorescence is a kind of short-lived light excited by the emission of light, and its frequency range is usually in the ultraviolet region. Due to the energy loss during the excitation process, the excitation wavelength will be longer than the incident wavelength, which is generally in the visible light region.
Figure 1. Schematic diagram of electronic transition

As shown in Figure 1.1, S0 and S1 represent the ground state and excited state, respectively, hvEX and hvEM represent the excitation and emission energy, respectively. After the electron absorbs the energy, it is excited from the original ground state to an unstable excited state, and then again Transition back to the ground state and release energy at the same time. The amount of energy released determines the fluorescence emission wavelength of the substance.

1.1.2 Overview of fluorescent probes
A fluorescent probe is an organic molecule that uses fluorescent signals to express the physical properties of the measured ion or molecule. It is generally composed of two parts, the acceptor part and the fluorophore. These signals change through different mechanisms to complete the test Sensing detection of substances.

This method of fluorescence analysis not only has a small sample size, good selectivity, and very high sensitivity. Now, fluorescence analysis has been widely used in many fields such as pharmacology, environmental science, biology, and information science.

1.1.3 Application of fluorescent probes in living cells in organisms
In 1914, the scientific experiment team studied fluorescent yellow dyes and produced related probes for detection in vivo. This invention greatly promoted the development of fluorescent probes in living cells. After that, the design and synthesis of new pH and mercury ion fluorescent probes and the new method of methyl pyran deposit site detection, fluorescent probes for in vivo detection have never stopped. With the discovery of amino acid dyes, scientists have a clearer understanding of various life activities in living cells.

For example, in recent years, scientists have produced a ratio-type upconversion nanoprobe for the detection of cells in organisms [1]. It uses multivalent hyaluronic acid as a universal ligand UCNPs (HA-UCNPs) for highly sensitive sensing and bioimaging of ROS and effective diagnosis. As the main component of synovial fluid and extracellular matrix, hyaluronic acid is a negatively charged natural glycan that is biocompatible and ROS scavenging means. Such a function, when applied to UCNPs, not only gives UCNPs colloid stability, but also biocompatibility and ROS recognition performance. At the same time, HA contains multiple functional groups conjugated to the main chain that can be used for UCL receptor chromophores. In addition, the cleavage of the HA backbone and the detachment of the chromophore are induced by ROS to abolish LRET in the HA fragment labeled with UCNPs and make its ratio UCL emission as a detection signal. It has high significance. This
novel nanoprobe has been proved to be able to early monitor the response of arthritic animals to MTX treatment with anti-arthritis drugs.

1.1.4 Application of Nano Probes in Rheumatoid Arthritis

Scientists [2] used samples of HA-UCNP nanocomposites incubated at 37°C with ROS produced by different chemistries and then analyzed the characteristics of the active oxygen reaction of HA-UCNP hybrids investigated by polyacrylamide gel electrophoresis (PAGE). Both hROS and $O_2^-$ will have obvious polymer fragments, while hydrogen peroxide has no significant effect on molecular weight. Under normal circumstances, hydrogen peroxide is shown alone, but the backbone HA is prone to cleavage in the polymer chain, resulting in very low activity after the B-scission reaction. It is worth noting that by further coupling suitable UCL to become a receptor, a ratio up-conversion nanoprobe can easily be established for ROS sensing. In particular, the probe will be more suitable for detecting active oxygen pools in live animals such as arthritic joints.

1.2 Application of fluorescent probe in pH detection

1.2.1 Overview of pH probe

At present, there are many methods for detecting pH [3]. Among them, the method of using probes, due to the high sensitivity and specificity of fluorescent probes, and can clearly show the changes of hydrogen ions in living cells after imaging, so it is widely used.

1.2.2 Application of pH probe in lysosomal pH detection

The pH value of the lysosome should meet at least three prerequisites during the test: one) it should be lysosome localized, and two) it should have a long analysis wavelength (greater than $L = 650$ nanometers or near infrared) to try reduce autofluorescence and biological damage, and c) It should have a reversible pH-dependent proportional response to avoid the influence of several variants such as concentration and length of optical path. Taking these criteria into consideration, we developed the pH of hemolysis. Using hemolysis pH-based fluorescence imaging, we found that the pH of the lysosome rises with increasing temperature, and this rise in pH is irreversible in living cells. The proportional response of hemolysis pH value matches well with the physiological pH range of lysosomes (pH 3.8-5.0), making it promising as a near-infrared fluorescent probe for accurate measurement of lysosome pH. These results indicate the effect of lysosome pH.

1.2.3 Classification of common pH fluorescent probes

Fluorescein derivatives: Fluorescein organic small molecules have longer fluorescence emission wavelength and high quantum yield. Fluorescein has become the preferred fluorescent probe for measuring pH in the neutral range. Scientists [4] modified these fluoresceins to increase various groups into fluorescein derivatives, which can be used in living cells for a long time. Imaging in solution.

Cyanine derivatives: cyanine derivatives [5], a classic near-infrared fluorescence probe material, which has been often used and developed to study imaging under different spectrosopes. Hemicyanine is derived from the decomposition of cyanine formed, usually with high stability. The excitation emission spectrum of cyanine derivatives is red-shifted to the near-infrared region, so that the emitted long-wavelength light has a better penetration effect, eliminating many fluorophores from being interfered by tissue fluorescence due to short-wave excitation, thus becoming the popular design direction of pH probes of a near infrared region in recent years.

1.3 Research and progress of pH fluorescent probes

1.3.1 Research on pH fluorescent probe

The pH in the cell body plays a key role in a series of tissue activities such as enzyme activity, cell proliferation and death, drug resistance, ion transmission, and muscle contraction. The pH in abnormal
cells can affect the function of normal cells in patients with common diseases such as cancer and Alzheimer's disease. This method of fluorescence detection is relatively simple compared with other methods of detecting pH, such as microelectrodes and nuclear magnetic resonance technology, and at the same time has high sensitivity. The qualitative detection of pH can usually be completed by the change of fluorescence intensity or the function of the switch, but the detection of this single index will be affected by many factors. Only by issuing a fluorescent probe with a ratio detection effect can it be better solve these problems.

1.3.2 Ratio fluorescent probe

Parker et al. designed and synthesized a ratio-type pH probe by combining the cell-permeable N-methylsulfone amine group and the macrocyclic Eu (III) complex. This molecule is characterized by a specific long wavelength. The probe's pA:a = 6.15, its detection range of intracellular pH is 6-8.

Bojinov et al. designed and synthesized a pH probe by covalently linking rhodamine and 1,8-naphthimide. Due to the FRET mechanism, 1,8-naphthimide is used as an energy donor. Rhodamine is an energy acceptor. Even better, FRET efficiency can reach 96-97%. The pKa of probe 39 is 5.06. In a neutral environment, molecule 39 has no fluorescence, which may be due to the fact that the rhodamine group is in a closed loop. The state may also be due to the quenching of the PET fluorescence of the energy donor 1,8-naphthimide. When the pH gradually decreases, the fluorescence slowly recovers, and the intensity can increase to more than 40 times the original.

1.3.3 Specificity for pH detection

Ions, proteins, reactive oxygen species, amino acids, and glucose have no obvious changes in the fluorescence signals detected in the presence of their physiological concentrations in these species, indicating that hemolytic pH is highly selective for pH detection. In addition, the effect of temperature from 318°C to 458°C was investigated by the fluorescence of the hemolytic pH itself. It was found that changing the tempering ATURE within this range did not significantly affect the fluorescence signal of the ratio of hemolytic pH. The pH value of hemolysis has good biocompatibility, and it can detect the minimum interference of pH changes in the lysosome with temperature and other biologically related species.

2. Experimental part

2.1 Experimental steps

Figure 2. Target compound (E)-5-carboxy-2-(4-hydroxy-styrene)-1, 3, 3-trimethyl-3H-Iodide Indole

2.1.1 Synthesis of intermediate 5-carboxy-1, 2, 3, 3-tetramethyl-3H-indole iodide

Accurately weigh 1g (4.92 mmol) of 4-carboxyphenylhydrazine hydrochloride and 394mg (4.92 mmol)
of sodium hydroxide into a 100 mL wedge-shaped flask, add appropriate amount of ethanol for ultrasonic dissolution, and use a glass rod at room temperature Stir and dissolve for 30 min.

Then spin the ethanol to dryness, transfer the remaining solids to a 100 mL three-necked flask, add 10 mL of glacial acetic acid to dissolve, then add 869 mg (10.6 mmol) of sodium acetate, and ultrasonically dissolve. Finally, add 855 µL (7.9 mmol) of 3-methyl-2-butanone, set up the reaction device, heat to 100°C, and stir at reflux for 16 h. After the reaction is complete, spin out the glacial acetic acid, cool to 0°C with ice water, slowly add saturated potassium carbonate solution until no bubbles are generated, adjust the pH to 4 with hydrochloric acid, and then extract the aqueous layer of the mixture with dichloromethane Three times, the oil phase was collected, dried with anhydrous sodium sulfate, filtered with suction, and then the dichloromethane was spun off, and a red oil was obtained after spin drying.

The red oil obtained above was dissolved in 19 mL of acetonitrile, and transferred to a 50 mL three-necked flask, added with 1500 µL of methyl iodide, heated to 45°C, and stirred for 12 h. After the reaction was cooled to room temperature, a large amount of diethyl ether was added until no precipitation occurred, filtered with suction and washed with diethyl ether three times to obtain an ochre solid, which was dried in vacuum to obtain a mass of 0.7136 g and a yield of 60%.

1H NMR: δH (400 MHz, DMSO-d6, Me4Si): 1.57 (s, 6H), 2.81 (s, 3H), 4.00 (s, 3H), 8.03 (d, 1H), 8.19 (d, 1H), and 8.39 (s, 1H).

13C NMR: δC (100 MHz, DMSO-d6): 199.48, 166.95, 142.42, 141.72, 132.04, 130.83, 124.68, 115.85, 54.72, 35.52, 21.96, and 15.12.

2.1.2 Synthesis of the final product compound 1
Weigh 500 mg (1.5 mmol) of the product from the previous step into a 50 mL three-neck round-bottomed flask, then add 139.1 mg (0.8 mmol) of 4-hydroxybenzaldehyde, add 15 mL of ethanol, pulverize by ultrasound, stir, and heat to 78 °C reflux for 12 h. Cooled, filtered with suction, was washed with frozen ethanol for several times, and dried in vacuum to obtain 0.3338 g of orange-red solid with a yield of 63.7%.

1H NMR: δH (400 MHz, DMSO-d6, Me4Si): 1.82 (s, 6H), 4.10 (s, 3H), 6.98 (d, 2H), 7.49 (d, 1H), 7.93 (d, 1H), 8.17 (t, 3H), 8.43 (t, 2H), 10.98 (s, 1H), and 13.32 (s, 1H).

13C NMR: δC (100 MHz, DMSO-d6): 183.81, 167.07, 164.32, 155.92, 145.68, 143.85, 134.60, 131.18, 130.87, 126.52, 124.19, 117.04, 115.12, 109.74, 52.26, 34.68, and 26.06. HRMS (EI) m/z: C20H20NO3I [M-I], 322.1442.

3. Results and inquiry

3.1 Characterization of the intermediate product 5-carboxy-1, 2, 3, 3-tetramethyl-3H-indole iodide
Spectrum analysis: According to the carbon spectrum analysis of the intermediate product, the peak position and the number of carbon atoms match the structural formula of the intermediate product, so the existence of the intermediate cyanine can be proved.

3.2 Characterization of the final product compound 1 ((E)-5-carboxy-2-(4-hydroxy-styrene)-1, 3, 3-trimethyl-3H-indole iodide)

3.2.1 Hydrogen spectrum characterization
Spectrum analysis: According to the hydrogen spectrum of the final product, perform hydrogen spectrum analysis. The position of the proton peak and the number of hydrogen atoms match the structural formula, which can prove the existence of the product.

3.2.2 Carbon spectrum characterization
Spectrum analysis: Carry out carbon spectrum analysis according to the carbon spectrum of the final product, and the product is in accordance with the structural formula.

3.2.3 Mass spectrometry analysis
The relative molecular mass of the molecule is 322.14, which is consistent, which proves the existence
of the product.

![Figure 3. Mass spectrum of the final product](image)

4. Conclusion and outlook
In this article, after the synthesis and characterization of the final product, the UV spectroscopy is prepared to observe the changes in the fluorescence spectrum in the pH titration. And through the study of the results, in-depth exploration of (E)-5-carboxy-2-(4-hydroxy-styrene)-1, 3, 3-trimethyl-3H-iodoindole cyanine derivatives the mechanism of fluorescence ratio probe in pH detection.

In future experiments, we will strengthen the synthesis and purification technologies as much as possible to obtain more and more reliable characterization data, so as to make the product more reliable and credible and increase its potential value.

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