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Synthesis of Fluorescein Aldehydes for the Sensitive Detection of L-Cysteine †

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Abstract: Amino acids containing thiol-groups such as Cysteine (Cys), Homocysteine (Hcy), and Glutathione play an important role in a great variety of biological processes. However, too low, but also too high concentrations can have negative effects on human health. Therefore, it is of great importance to sensitively detect these risk factors for cardiovascular and neuronal diseases. Furthermore, Cys is an essential amino acid for the growth of pathogenic water-borne bacteria, like *Legionella* sp. Hence, a reliable, sensitive and selective Cys-detection method that is incorporated into automated biosensors would represent a great tool for a broad range of biological and medical applications. We describe the simple synthesis of fluorescein aldehyde probes for the detection of biologically important thiols, focusing on Cys. The probes displayed highly sensitive responses to L-Cysteine hydrochloride monohydrate in the range of their physiologically relevant levels in the visible and UV spectral region.

Keywords: fluorescein aldehydes; L-Cysteine; detection

1. Introduction

Elevated levels of amino acids Cys and Hcy have shown to be associated with an increased risk of myocardial infarction, stroke, and venous thromboembolism. In the case of Cys, neurotoxic effects have been demonstrated in vitro and in vivo [1,2]. However, a lack of Cys can induce several health disorders such as slowed growth, muscle and fat loss, hair depigmentation, and liver or skin damage. Therefore, our goal was the development of a simple and sensitive detection method for Cys to be used in the field of bio-medical applications [3].

2. Materials and Methods

2.1. Chemical Synthesis

Targeted compounds (1) Fluorescein Monoaldehyde (MOAF) and (2) Fluorescein Dialdehyde (DIAF) were synthesized by a Reimer-Tieman formylation reaction of fluorescein (Figure 1) [4,5]. Briefly, sodium hydroxide (125 mM, 16.0 equiv) was slowly added to a fine suspension of fluorescein (7.52 mM, CAS: 2321-07-5) in methanol. After complete dissolution of fluorescein, chloroform (37.5 mM, 15 equiv) and 15-Crown-5 (0.3760 mM, 0.1 equiv) were slowly added with aid of syringe. The resulting dark brown clear solution was vigorously stirred at 55 °C in an oil bath for 42 h. After cooling, the reaction mixture was poured on stirred ice cold 1 M H_2SO_4 with aid of minimal amount of distilled water. After acidification (pH 1–2) the crude product quickly precipitated. The solid was

filtered off and washed with ice cold water. The wet filtration cake was frozen and lyophilized to afford dark yellow-brown powder. The crude product was purified by flash column chromatography on silica (0% to 50% EtOAc in petroleum ether) to remove major part of unreacted fluorescein. The mixture of DIAF and MOAF aldehydes was re-purified by a second flash column chromatography on silica (0% to 50% EtOAc in CH_2Cl_2) to yield DIAF (white-beige solid, 20 mg, 0.7%) and MOAF (yellow solid, 94 mg, 3.5%). Synthesized aldehydes were clearly identified by FTIR analysis (4000–650 cm^{-1}, Perkin Elmer FT-IR Spectrometer Spectrum Two equipped with Attenuated Total Reflection (ATR) mode using ZnSe as the optical material) [2]. Furthermore, on TLC analysis, aldehydes DIAF and MOAF gave active spots under UV (365 nm), $KMnO_4$ and 2,4-DNP (2,4-dinitrophenylhydrazine hydrochloric acid solution in ethanol) stains. Compared to the synthesized aldehydes, fluorescein had a lower R_f and was not 2,4-DNP active.

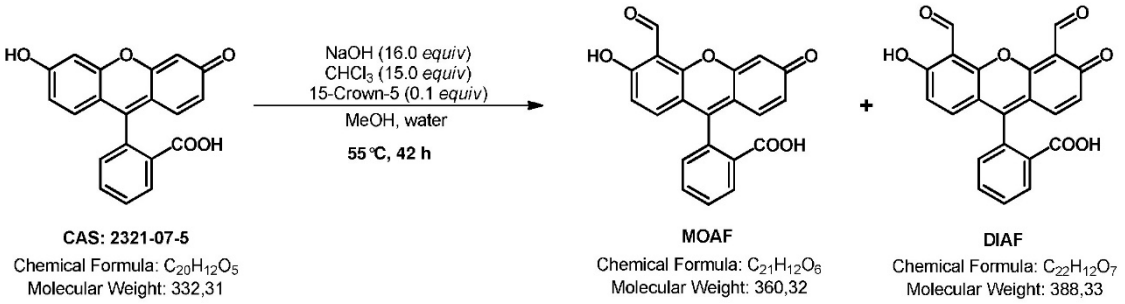

Figure 1. Reimer-Tieman formylation of fluorescein.

We were able to separate desired aldehydes from the crude reaction product by flash column chromatography on silica. Synthesized aldehydes were clearly identified by FTIR analysis. Furthermore, DIAF and MOAF TLC spots (comparing to fluorescein, control spot) were active towards 2,4-DNP TLC stain; proof reagent of aldehydes and ketones.

2.2. Cys Measurements

Two different methods were used for determination of various L-Cysteine hydrochloride monohydrate (CAS: 7048-04-6) and L-Cys (CAS 52-90-4) concentrations. Therefore, a dilution series of Cys in water was prepared. First, visual analysis of the behavior of mixtures of MOAF (1 mM) or DIAF (0.5 mM), respectively, together with Cys (0.5 and 5 mM) under visible and UV light (254 and 365 nm) using a Vilber Lourmat VL-6.LC UV lamp was done (Figure 2). Secondly, fluorescence measurements with a Perkin Elmer LS55 Luminescence Spectrometer were performed with mixtures of Cys concentrations ranging from 1 μM to 1 mM with 2.5 μM DIAF. The solutions were excited at 460 nm and emission spectra were measured from 470 to 650 nm (Figure 3).

3. Results and Discussion

We find that upon addition of L-Cysteine (CAS: 7048-04-6; 0.5–5 mM) to a solution of MOAF (1 mM) and DIAF (0.5 mM), a color change from bright yellow to colorless was observed (Figure 2c,f). Higher Cys concentrations (5 mM) led to immediate color reactions whereas for lower concentrations (0.5 mM) several minutes of incubation were necessary. Additionally, fluorescence quenching of MOAF and DIAF solutions with Cys were detected (Figure 2a,b,d,e). DIAF showed to be more sensitive for the L-Cysteine hydrochloride monohydrate detection then MOAF. Fluorescence quenching and color bleaching happened immediately for both tested concentrations (Figure 2e,f). In future experiments a wider concentration range of Cys samples will be analyzed to further evaluate the optical detection limits of this technique.

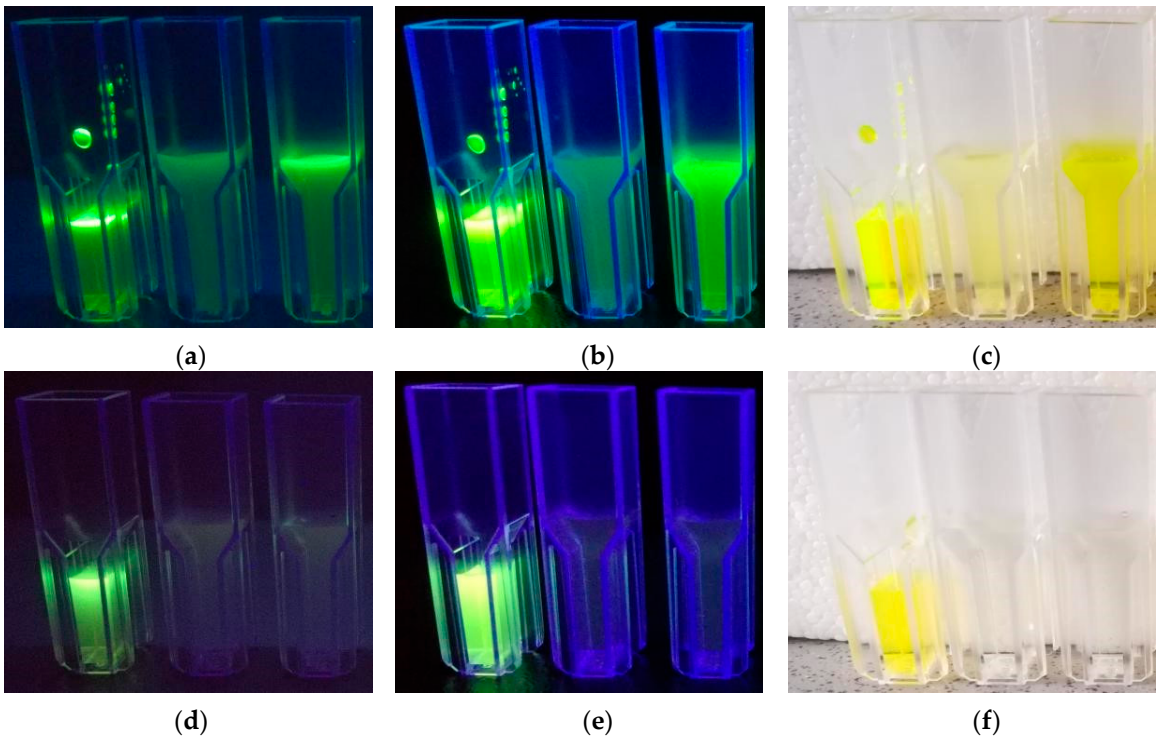

Figure 2. Color changes of solutions of MOAF (**a**–**c**) and DIAF (**d**–**f**) with L-Cysteine under UV-light at 254 nm (**a**,**d**), 365 nm (**b**,**e**) and visible light (**c**,**f**). The left cuvette shows sole MOAF (1 mM)/DIAF (0.5 mM), respectively, the middle cuvette MOAF/DIAF mixed with 5 mM L-Cysteine and the right cuvette MOAF/DIAF commingled with 0.5 mM L-Cysteine hydrochloride monohydrate. The usage of DIAF for Cys detection delivered faster results for the color changes and fluorescence quenching.

The fluorescence measurements of DIAF together with L-Cys hydrochloride monohydrate are shown in Figure 3. A clear correlation between fluorescence emission intensity of DIAF and L-Cys hydrochloride monohydrate concentrations in water (pH 9.5) could be observed. The addition of Cys solutions to DIAF lowered its fluorescence emission spectrum within the range from 490 to 540 nm. The lower the Cys concentration the higher signal peaks could be detected.

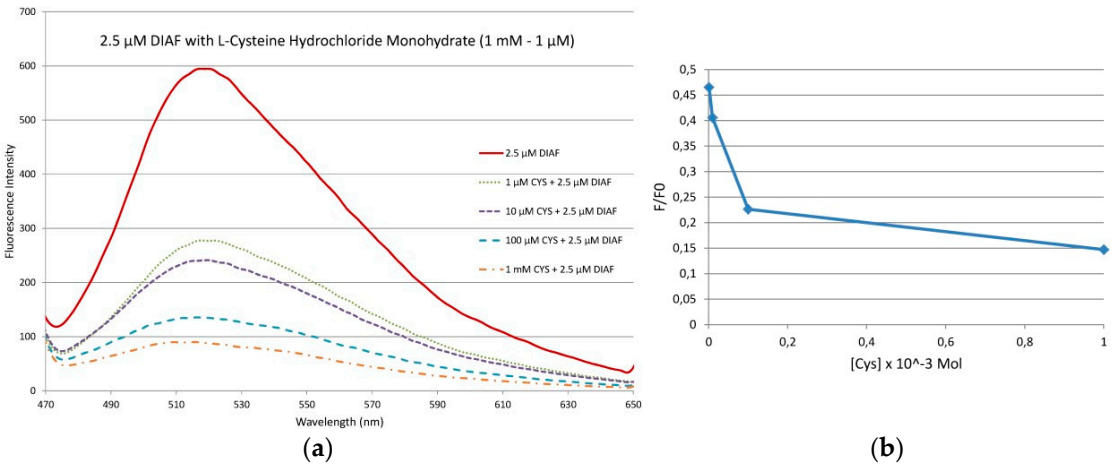

Figure 3. (**a**) Fluorescence emission spectra of Fluorescein Dialdehyde (2.5 µM) and L-Cysteine hydrochloride monohydrate (1 mM, 100 µM, 10 µM and 1 µM) excited at 460 nm in water, pH 9.5 at room temperature. The fluorescence intensity of DIAF decreases with increasing Cys concentrations. (**b**) F0 represents the maximum fluorescence intensity in the absence of analyte and F represents the corresponding intensity in the presence of analyte.

In conclusion Fluorescein Aldehydes proofed to be powerful tools to determine L-Cysteine at very low levels. The presented methodology, especially by using DIAF, shows great promise for fluorescence and UV-vis detection of aminothiols in biological samples. In future experiments we aim to evaluate the cross-reactivity of DIAF solutions with other amino acids, and the exact limits of Cys-detection, and to test its applicability for bio-medical application such as *Legionella* detection in water samples.

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Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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