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Photophysical study of benzanthrone 3-isothiocyanate as novel fluorescent label

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In modern biochemical research, much attention is paid to the development and synthesis of new fluorescent markers used for protein labeling. Reaction with an isothiocyanate group is one of the most widely used methods for fluorescent labeling of amino acids and proteins. In the present work, an isothiocyanate derivative of benzanthrone was obtained and characterized as a possible fluorescent marker. The interaction of 3-isothiocyanatobenzanthrone with a number of natural amino acids was studied. The study also examined the processes of conjugation of the synthesized isothiocyanate with albumin, pepsin and gelatin. The photophysical parameters of the obtained conjugates were studied in detail. Considering the fluorescent properties of the obtained conjugates, the isothiocyanate under study appears promising for fluorescent labeling purposes.

Keywords: Luminescent label; Benzanthrone; Isothiocyanate; Amino acids; Proteins; Fluorescence

INTRODUCTION

Among numerous imaging and detection methods, the fluorescence approach shows significant advantages in terms of ease of fabrication, low cost, and high sensitivity, which has been widely used to study various biomolecules. It is important to develop and synthesize fluorescent markers with good biological activity and optical properties. Therefore, new labels and new methods are constantly being developed [1, 2]. In modern research, much attention is paid to the development and synthesis of new fluorescent markers used for labeling amino acids, peptides and proteins [3, 4]. This includes molecular design considering the best choice of a reactive group in the fluorophore structure through which conjugation to the target biomolecule occurs. The procedure for introducing active groups into the fluorophore structure is used to obtain the necessary spectral properties of conjugates for their further detection and analysis [5, 6].

Typically, direct chemical labeling of proteins targets the amino and thiol groups. The most commonly used amine-reactive markers include succinimidyl ethers, imidoethers, sulfonyl chlorides, and isothiocyanates [6-8]. Fluorescein- and rhodamine-derived isothiocyanates are among the most popular conjugation reagents for amine modification during protein labeling [9-11].

In this research we designed a new potential fluorescent marker for proteins, isothiocyanate-functionalized benzanthrone derivative with a target NCS group at position 3 of the aromatic core. 3-Substituted benzanthrone derivatives are compounds with excellent emissive properties, making these luminescent dyes find numerous applications in biomolecular research and bioimaging, mainly as non-covalent labels [12-15]. Further in the present work, the reactions of the newly obtained 3-isothiocyanobenzantrone with a number of amino acids were studied in order to find out the applicability of this isothiocyanate for the labeling of amino acids. The developed marker was also applied in bioconjugation reactions with proteins.

EXPERIMENTAL

All reagents, solvents, amino acids and proteins were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

The progress of the synthesis and the purity of substances were controlled by thin layer chromatography (TLC) using silica gel plates (Fluka F60254, 20×10, 0.2 mm) with toluene as eluent. Melting points were obtained on an MP70 Melting Point System apparatus.

Fourier-transform infrared (FT-IR) spectroscopy measurements were carried out by a Bruker Vertex 70v vacuum spectrometer equipped with an attenuated total reflection accessory. ^{1}H NMR spectra were registered by a Bruker equipment at a frequency of 500 MHz in $CDCl_3$ (with tetramethylsilane as internal standard) at ambient temperature.

Shimadzu GCMS-QP2010 system (Shimadzu Corporation, Kyoto, Japan) was used for the analysis. The gas chromatograph was equipped with an electronically controlled split/splitless injection port and a 5% diphenyl-/95% dimethylpolysiloxane

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fused-silica capillary column (Rtx-5SIL-MS, 30 m×0.32 mm, 0.25 μm film thickness). Mass spectra (MS) were registered by a mass spectrometer in electron ionization mode (ionization energy of 70 eV). Detection was realized in scanning mode within the range of m/z 35–500.

Absorption spectra were recorded on a UV–visible spectrophotometer Specord UV/VIS. Fluorescence spectra were obtained using FLSP920 (Edinburgh Instruments Ltd.) spectrofluorometer in the spectral range of 430–800 nm. Emission measurements were performed in quartz cells with an absorbing layer thickness of 1 cm.

Synthesis

3-Aminobenzanthrone (**1**) was synthesized by nitration of benzanthrone and subsequent reduction of the 3-nitroderivative according to the literature procedures [16, 17].

- *Synthesis of 3-isothiocyanatobenzanthrone (2).* In a 25 ml round-bottom flask, a mixture of 3-aminobenzanthrone (0.25 g, 1 mmol), carbon disulfide (0.50 g, 6.5 mmol), N,N'-dicyclohexylcarbodiimide (0.25 g, 1.2 mmol) and pyridine (7 ml) was stirred in an ice bath at 0°C for 4-5 h. Then, 0.10 g of DCCD was added and the resulting mixture was continuously stirred at ambient temperature during 12-14 h. The progress of ongoing reaction was monitored by TLC. The pyridine was then evaporated and the reaction mixture is dissolved in 20-30 ml of ethyl acetate. The product is purified by column chromatography on a 1.5 × 30 cm column packed with silica gel 40/100 (eluent: dichloromethane) to give a yellow solid in 60% yield, m. p. 200-201ºC. FT-IR spectra, λ_{max} (KBr), cm^{-1}: 2925, 2851, 2120, 1661, 1627, 1572, 1553, 1505, 1448, 1347, 1229, 1087, 1044, 892, 772, 641. NMR (500 MHz, $CDCl_3$) δ 8.83 (dd, *J*=7.3; 1.3 Hz, 1H), 8.47-8.57 (m, 2H), 8.39 (d, *J*=7.2 Hz, 1H), 8.29 (d, *J*=7.8 Hz, 1H), 7.92 (dd, *J*=8.3; 7.3 Hz, 1H), 7.79 (ddd, *J*=8.1; 7.2; 1.6 Hz, 1H), 7.49–7.67 (m, 2H). MS (*m/z*): 287 [M+] (100), 259 (15), 227 (14), 200 (17), 129 (11), 100 (11).
- *Conjugation with amino acids.* A freshly prepared solution of 3-isothiocyanatobenzanthrone in anhydrous DMSO (0.3 mL with concentration of 3 mg/mL) was poured dropwise with continuous stirring to an appropriate amino acid solution (4 mL, 1 mg/mL) in 0.1M bicarbonate buffer (pH 9.3). The prepared mixture was protected from light and stored at ambient temperature for 0.5 h with constant slow stirring. The solution should be left for 8 h at 4ºC without light.
- *Conjugation with proteins.* A freshly prepared solution of 3-isothiocyanatobenzanthrone in anhydrous DMSO (0.1, 0.2 or 0.3 mL with concentration of 1 mg/mL) was poured dropwise with continuous stirring to an appropriate protein solution (4 mL, 2 mg/mL) in 0.1M bicarbonate buffer (pH 9.3). The reaction mixture was protected from light and stored at ambient temperature for 0.5 h with constant slow stirring. Then the reaction mixture was incubated at 4ºC for 12 h.

RESULTS AND DISCUSSION

Synthesis and characterization of the new label

Isothiocyanate group is a very useful building block in synthetic chemistry for the preparation of both sulfur- and nitrogen-containing organic compounds [18-20]. Owing to their synthetic and biological importance, numerous methods for the preparation of isothiocyanates from amines, dithiocarbamates, organic halides, nitriles, olefins, aldoximes, etc. have been developed [21].

In the present research we used 3-aminobenzanthrone for synthesis of the appropriate isothiocyanate (see Fig. 1). This method involves converting the amine into appropriate isothiocyanate in a solution of pyridine in the presence of an aqueous binder – N,N-dicyclohexylcarbodiimide is a highly versatile dehydrating reagent. During the reaction, the color of the solution changed from red to orange.

Monitoring the progress of the isothiocyanate synthesis in the reaction mixture by TLC shows the formation of a new yellow product with a bright green luminescence, in contrast to the initial amine, which has a red color and emission. The structure of the obtained derivative **2** was confirmed by FT-IR, NMR and mass spectrometry data.

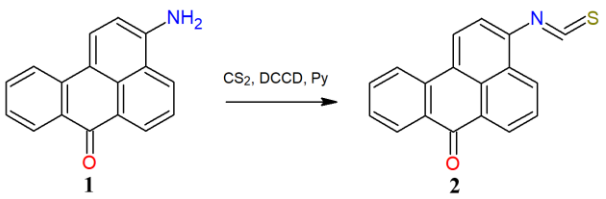

Fig. 1. Synthesis of 3-isothiocyanatobenzanthrone (**2**).

The ^1H NMR spectrum contains only multiplet signals (from δ 7.50 to 8.85 ppm) of nine aromatic hydrogen atoms**,** which is typical for 3-substituted benzanthrone derivatives [22].

In the IR spectrum, characteristic vibrations of the isothiocyanate group are observed at 2120 cm^{-1}, which also confirms the obtaining of the target substance.

The spectral absorption and emission data of the synthesized isothiocyanate solutions in various organic solvents are summarized in Table 1.

Positions of absorption maxima are situated between 425–435 nm. The obtained compound is fluorescent in solutions in the region of 500–560 nm, showing a hypsochromic shift of 100–130 nm compared to the parent amine **1** and many 3-substituted amino and amidino derivatives, which emit in the red region [23]. In addition, the Stokes shifts are smaller than those for amines and amidines.

Conjugation with amino acids and proteins

In the present research the conjugation with seven natural amino acids (glycine, L-alanine, L-oxyproline, L-aspartic acid, L-cysteine, L-methionine and L-arginine) and with three proteins (bovine serum albumin, porcine pepsin and gelatin) was studied. For conjugation with amino acids the literature procedure for known isothiocyanates was applied [5, 6].

It is well known that the isothiocyanate group can react with thiol and amino groups present in amino acids and proteins by forming appropriate C-S or C-N covalent bond [24, 25]. Two possible ways of interaction of the synthesized isothiocyanate **2** with the mentioned reactive groups are shown in Fig. 2.

The spectral properties of the obtained amino acids conjugates were investigated. As can be seen from the results obtained (Fig. 3), the reaction of all amino acids with the marker produces fluorescent products, the emission of which significantly exceeds the luminescence of the original compound. The largest increase in emission of 27 times was observed for the arginine conjugate. The conjugates of methionine, glycine and alanine showed a 20-23 times increased emission. The smallest increase in emission was observed for cysteine and aspartic acid conjugates. Differences were also observed in the location of the fluorescence maxima of the conjugates compared to the starting isothiocyanate. The fluorescence maxima of the conjugates of arginine, methionine, glycine and alanine are shifted hypsochromically by 15-20 nm, the fluorescence of the oxyproline conjugate is red-shifted by 20 nm compared to compound **2**, and for derivatives of cysteine and aspartic acid the positions of the maxima do not change.

Table 1. Absorption and emission properties of compound **2** in 10^{-5} M solutions.

Solvent	Absorption λ_{abs}	lgε	Fluorescence λ_{em}	Stokes shift, cm^{-1}
Hexane	425	4.23	498	3449
Benzene	431	4.21	520	3971
Chloroform	433	4.23	520	3864
Ethyl acetate	425	4.23	552	5413
Acetone	423	4.24	543	5224
Ethanol	424	4.19	538	4998
Dimethylformamide	426	4.20	556	5489
Dimethylsulfoxide	432	4.21	552	5032

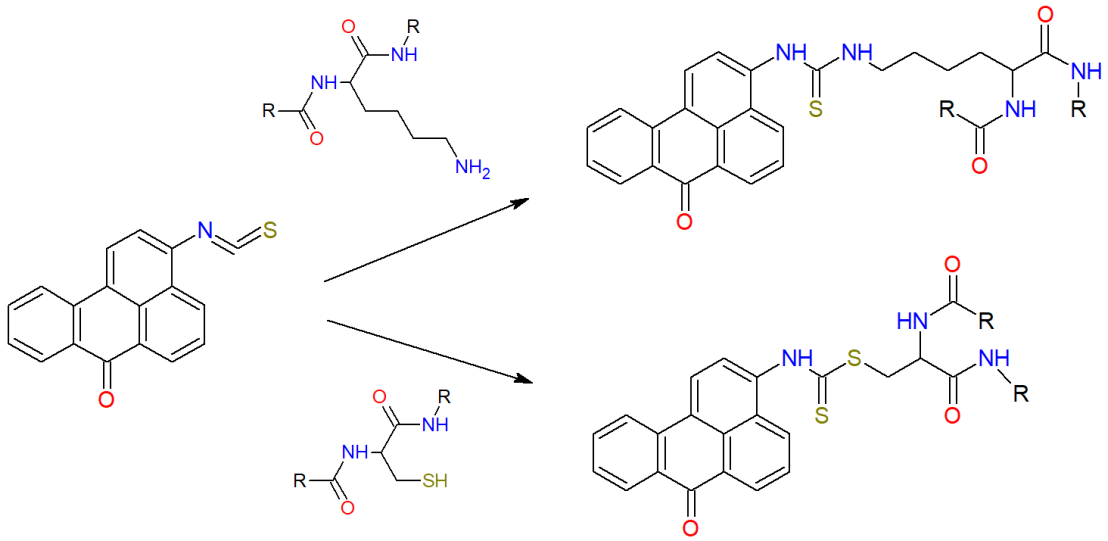

Fig. 2. Interaction of 3-isothiocyanatobenzanthrone with nucleophilic groups: with an amino group, forming thiourea (top); with a thiol group, giving dithiocarbamate (bottom).

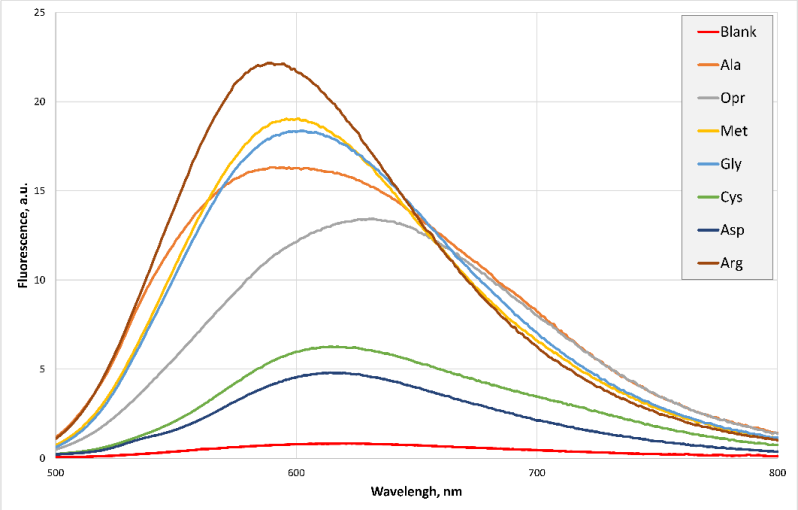

Fig. 3. Fluorescence spectra of compound **2** (blank) and its amino acid conjugates.

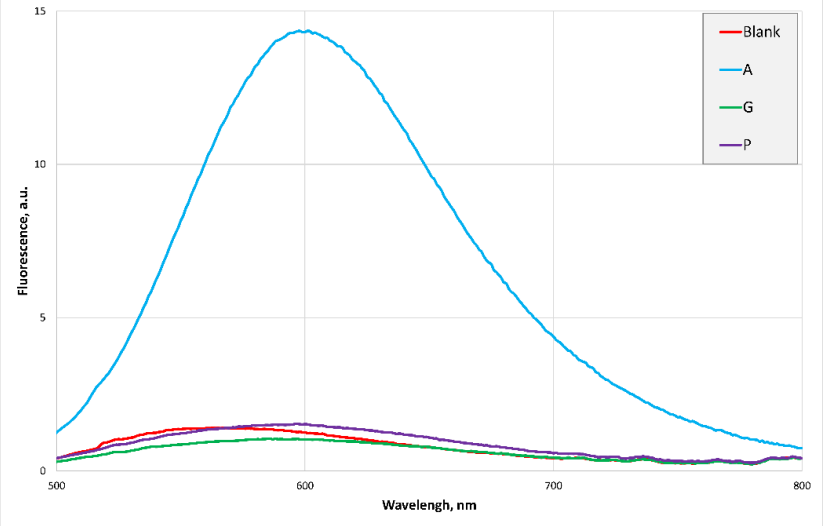

Fig. 4. Fluorescence spectra of compound **2** (blank) and its pepsin (P), gelatin (G) and albumin (A) conjugates.

Developed marker **2** was then used in bioconjugation reactions with three proteins at different isothiocyanate:protein ratios (0.1:8, 0.2:8 and 0.3:8 mg).

The most pronounced changes in the photophysical properties of the obtained protein conjugate compared to the initial isothiocyanate are observed for albumin (see Fig. 4). While the emission of gelatin and pepsin conjugates is almost the same as that of the starting compound **2**, the luminescence intensity of the albumin conjugate turned out to be 10 times higher than the isothiocyanate emission. A common feature of all conjugates is a bathochromic shift of the luminescence maximum by 20-30 nm compared to the initial marker.

Undoubtedly, the differences in the properties of the obtained conjugates are associated with their composition and structure. Bovine serum albumin is a globular protein which consists of 583 amino acid residues (including 35 cysteine, 60 lysine, 26 arginine residues, etc.) in a single chain with a molecular weight of 66 kDa [26, 27]. Pepsin is a gastric aspartic proteinase with a molecular weight of 3.4 kDa containing 327 amino acid residues in a single polypeptide chain [28, 29]. Gelatin is a product of collagen destruction, its molecular weight is about 100 kDa, it contains practically no cysteine and contains a small amount of lysine compared to albumin [30]. Apparently, albumin not only contains quite a lot of amino acid residues that react with the isothiocyanate group, but these residues are also located on the outside of the albumin globule, which results in the formation of a highly emissive conjugate.

CONCLUSION

3-Isothiocyanatobenzanthrone was designed as a fluorescent probe for amino acids and protein bioconjugation, and its application was tested using

bovine serum albumin, porcine pepsin and gelatin as model proteins. The highest emission response was observed for albumin, what can later be used for studying the biochemical processes in which this protein is involved. Thus, it can be summarized that the isothiocyanate developed and studied in this research appears promising for fluorescent labeling purposes.

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