The methodology of the synthesis of the modified fluoresceins for the click reactions was developed. Proposed synthetic protocol allows to increase the yield of the final and intermediate compounds and to optimized the procedure of their isolation and purification.

Key words: modified fluorescein, fluorescein, click reaction.

INTRODUCTION. Reactions of azide-alkylcicloaddition are well known since 1893. However, after discovering cupper-promoted azid-alkilic cycloaddition [1, 2] this type of reactions gained new life. Due to the high yields, mild reaction temperatures, selectivity of the process and variability of suitable solvents this type of reaction became integral part of click-chemistry.

At the same time, with the elaboration of click chemistry, the techniques of labelling biological objects, particularly by fluorescent dyes, were widely developed. The use of fluorescent labels provides the ability to visually monitor the process streamlines hardware load and reduces research time [4]. Also, unlike most radioisotope probes, the vast majority of fluorescent dyes retain their properties over time. These factors have made fluorescence labeling one of the most widely used analytical methods in biochemistry, biology and medicine.

Besides the use for synthesis of the new biologically active compounds [5], azide-alkyne cyclic coupling reaction due to its mild conditions allows the modification of macromolecules in vivo. For example, cupper-free click labeling and chondrocyte tracking in vivo is reported in [6].

Fluorescein is among the fluorophores mostly used for labelling of biomolecules due to its high quantum yields and good stability in biological media [7]. However, despite of commercial availability of...
fluorescein derivatives functionalized for use in click reactions, methods for their synthesis are virtually absent in the literature.

Therefore, we have developed a reliable and effective methodic for the synthesis of functionalized fluoresceines for the use in the click reactions.

**EXPERIMENT AND DISCUSSION OF THE RESULTS.** Synthesis of diacetyl N-(4-azidobutyl)-fluoresceine-5(6)-carboxamide (6) was performed in six stages, starting form resorcinol and trimellitic acid anhydride. The diacetylated analogue was synthesized for the click modifications in the “soft” conditions, since N-(4-azidobutyl)-fluorescein-5(6)-carboxamide (5) is poorly soluble in the classic organic solvents.

5(6)-carboxyfluorescein (1) was obtained by the reaction of resorcinol and trimellitic anhydride in methane sulfonic acid at 110°C. During the reaction equimolar mixture of 5th and 6th isomers is forming. Precipitation from water and crystallization from the different solvents gave insufficient result in separation of these two isomers. Cause it similar spectral properties, it was decided to work with the equimolar mixture of two isomers.

Diacetyl 5(6)-carboxyfluoresceine (2) was obtained by the refluxing of fluorescein (1) with acetic anhydride in pyridine (Fig. 1).

Synthesis of the azides was performed without excess of light and during purification temperature maintained below 35°C. All azides were used in the next step with an excess and without purification. 1,4-diazidobutane (3) was obtained by the standard reaction of 1,4-dibromobutane with an excess of sodium azide in dry dimethylformamide. Reaction was controlled by gas chromatography. After reaction was complete, reaction mixture was diluted with water and extracted with diethyl ether. Organic fraction was evaporated under vacuum without heating and diluted with tetrahydrofuran. Solution of 1,4-diazidobutane (3) was used in the next step without purification.

1-amino-4-azidobutane (4) was obtained by the reduction of azide group of 1,4-diazidobutane (3) with triphenylphosphine. Triphenylphosphine was added portion wise upon cooling. An excess of 1,4-diazidobutane (3) was used in order to prevent formation of 1,4-diaminobutane. Reaction was controlled by gas chromatography (Fig. 2).
Synthesis of modified fluoresceine for click reactions

N-(4-azidobutyl)-fluoresceine-5(6)-carboxamide (5) was obtained by the reaction of the diacetyl 5(6)-carboxyfluoresceine (2) with 1,1'-carbonyldimidazole (CDI) and an excess of 1-amino-4-azidobutane (4). The partial hydrolysis of acetyl group during the synthesis was noticed. Hydrolysis of acetyl groups with an excess of ammonia water solution was performed in order to simplify the purification.

Diacetyl N-(4-azidobutyl)-fluoresceine-5(6)-carboxamide (6) was obtained by the refluxing of fluorescein (5) with acetic anhydride in pyridine. (Fig. 3).

![Image](image_url)

Fig. 3: Synthesis of diacetyl N-(4-azidobutyl)-fluoresceine-5(6)-carboxamide (6)

Same synthesis could be performed without protection with acetyl group. However, it’s easier to work with acetylated fluorescein derivative.

We prefer to use acetylated fluorescein because it is nicely soluble in classic organic solvents, this simplifies further handling with labeled compounds. Acetyl group could be easily hydrolyzed by using of classic methods.

For the fluorescein (5) UV-Vis and fluorescence spectra were registered. Its absorbance maximum wavelength is located at 494 nm and fluorescence maximum is located at 520 nm (Fig. 4), that is similar to corresponding maxima of non-functionalized uranin (500 nm and 521 nm correspondingly [8]).

5(6)-carboxyfluorescein (1): trimellitic acid anhydride (10 g, 52.0 mmol) was dissolved in methane sulfonic acid (50 ml) and resorcinol (12 g, 109.3 mmol, 2.1 equiv) was added. The reaction mixture was stirred at 110 °C for two hours. At the end of the reaction, the reaction mixture was cooled and poured into water, the precipitate was filtered, crystallized from water and dried in air at 100 °C to obtain 5(6)-carboxyfluorescein (16.4 g, 44 mmol, 84%), which was used without purification in the next step.

Diacetyl 5(6)-carboxyfluorescein (2): 5(6)-carboxyfluorescein (10 g, 26.6 mmol) was dissolved in 50 ml of dry pyridine and acetic anhydride (10 ml, 106.21 mmol, 4 equiv.) was added drop wise at room temperature and reaction mixture was stirred at room temperature overnight. At the end of the reaction, the mixture was evaporated, dissolved in ethyl acetate (100 mL) and washed with 1 M HCl (3×50 mL), water (100 mL) and Brine (100 mL) solutions, dried over sodium sulfate and evaporated to dry in vacuum to give diacetyl 5(6)-carboxyfluorescein (12.2 g, 26.6 mmol, 100%). \(^1\)H NMR (400 MHz, DMSO-d$_6$) δ: 8.47 (dd, \(J = 1.5, 0.8\) Hz, 1H), 8.33 (dd, \(J = 8.0, 1.5\) Hz, 1H), 8.28
Fig. 4: Normalized UV-Vis (a) and fluorescence spectra (b) of N-(4-azidobutyl)-fluorescein-5(6)-carboxamide (5)

(dd, J = 8.0, 1.3 Hz, 1H), 8.15 (dd, J = 8.0, 0.8 Hz, 1H), 7.81 (dd, J = 1.3, 0.8 Hz, 1H), 7.48 (dd, J = 8.1, 0.7 Hz, 1H), 7.28 - 7.20 (m, 3H), 6.98-6.86 (m, 7H), 2.29 (s, 12H).

1,4-diazidobutane (3): 1,4-dibromobutane (20 ml, 167.5 mmol) was dissolved in dry dimethylformamide (100 ml) and sodium azide (43.54 g, 669.9 mmol, 4 equiv.) was added. The reaction mixture was stirred overnight at 55 °C (control by gas chromatography), cooled to room temperature, poured into 300 ml of water and extracted with diethyl ether. The organic extract was evaporated without heating on a vacuum pump. The reaction mixture was used in the next step without purification. Quantitative yield.

1H NMR (400 MHz, Chloroform-d) δ 3.32 (td, J = 5.1, 3.9, 2.2 Hz, 4H), 1.73 – 1.62 (m, 4H).

1-amino-4-azidobutane (4): Freshly prepared 1,4-diazidobutane (167.5 mmol) was dissolved in tetrahydrofuran (200 ml), the reaction mixture was cooled in an ice bath and triphenylphosphine (13.2 g, 50.25 mmol, 0.3 eq.) was added in small portions with control of gas evolution. Upon completion of the addition, the reaction mixture was gradually brought to room temperature and stirred overnight. Product formation was monitored by gas chromatography. At the end of the reaction, the reaction mixture was diluted with 200 ml of 2N HCl and tetrahydrofuran was evaporated under vacuum. The aqueous fraction was extracted with dichloromethane (3 × 100 ml) basified with 25% aqueous NaOH and extracted with diethyl ether (3 × 100 ml), dried over sodium sulfate and evaporated without heating on a vacuum pump. After evaporation, 1-amino-4-azidobutane is diluted with dry tetrahydrofuran to obtain a 25% solution which was used without purification in the next step. Quantitative yield.

1H NMR (400 MHz, DMSO-d6) δ 3.30 (t, J = 6.9 Hz, 2H), 2.79 (bs, 2H), 2.54 (m, 2H), 1.60 – 1.44 (m, 2H), 1.49 – 1.29 (m, 2H).

N-(4-azidobutyl)-fluorescein-5(6)-carboxamide (5): Diacetyl 5(6)-carboxyfluorescein (5 g, 10.86 mmol) was dissolved in dry tetrahydrofuran (50 ml) and 1,1'-carbonyldiimidazole (CDI) (1.93 g, 11.94) was added portion wise. (1.93 g, 11.94 mmol, 1.1 equiv.). The reaction mixture was stirred at room temperature overnight and a solution of 1-amino-4-azidobutane (4.95 g, 43.44 mmol, 4 equiv.) in tetrahydrofuran (20 ml) was added at once...
with vigorous stirring. The reaction mixture was stirred for 2 hours, evaporated to dry and diluted with 50 ml 1N HCl. The aqueous fraction was extracted with dichloromethane (3 × 25 ml) and the organic fraction was basified with 25% ammonia solution upon vigorous stirring. After a few minutes, the reaction mixture was neutralized with concentrated hydrochloric acid, the precipitate was filtered and dried on a vacuum pump to obtain N-(4-azidobutyl)-fluorescein-5(6)-carboxamide (4.25 g, 9.01 mmol, 83%). \( ^1 \)H NMR (400 MHz, DMSO-\( d_6 \)) \( \delta \) 10.13 (bs, 2H), 8.87 (t, \( J = 5.7 \) Hz, 1H), 8.49 – 8.44 (m, 1H), 8.25 (dd, \( J = 8.0, 0.7 \) Hz, 1H), 7.38 (dd, \( J = 8.0, 0.7 \) Hz, 1H), 6.72 (d, \( J = 2.1 \) Hz, 2H), 6.63 – 6.52 (m, 4H), 3.44 – 3.30 (m, 4H), 1.62 (h, \( J = 3.6 \) Hz, 4H).

**Diacetyl N-(4-azidobutyl)-fluorescein-5(6)-carboxamide (6):** N-(4-azidobutyl)-fluorescein-5(6)-carboxamide (4.25 g, 9.01 mmol) was dissolved in 50 ml dry pyridine and acetic anhydride (3.4 ml, 36.04 mmol, 4 equiv.) was added drop wise and reaction mixture was stirred at room temperature overnight. At the end of the reaction, the mixture was evaporated, dissolved in ethyl acetate and washed with 1M HCl (20 mL), water and Brine solution, dried over sodium sulfate and evaporated to dryness in vacuo to give diacetyl N-(4-azidobutyl)-fluorescein-5(6)-carboxamide (4.96 g, 9.01 mmol, 100%). \( ^1 \)H NMR (400 MHz, Chloroform-\( d_6 \)) \( \delta \) 8.33 (dd, \( J = 1.6, 0.8 \) Hz, 1H), 8.14 (dd, \( J = 8.0, 1.6 \) Hz, 1H), 7.22 (dd, \( J = 8.0, 0.7 \) Hz, 1H), 7.05 (dd, \( J = 2.1, 0.5 \) Hz, 2H), 6.79 – 6.68 (m, 4H), 3.48 (q, \( J = 6.4 \) Hz, 2H), 3.28 (t, \( J = 6.3 \) Hz, 2H), 2.25 (s, 6H), 1.80 – 1.55 (m, 4H).

**CONCLUSIONS.** Functionalized fluoresceines modified for click reactions were synthesized by the developed reliable protocol. Both 5\(^{th}\) and 6\(^{th}\) fluorescein isomers are applicable for the click reactions, dependent of the required reaction conditions. Final diacetyl N-(4-azidobutyl)-fluorescein-5(6)-carboxamide (6) was obtained in 6 steps synthesis with total yield more than 70%. After click reaction acetyl groups of this dye could be easily hydrolyzed by classic methods.

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щени виходи описаних стадій синтезу та оптимізовано способи виділення та очищення кінцевих та проміжних сполук.

Ключові слова: флуоресцений, азидзаміщені флуоресцени, клік реакції.

СИНТЕЗ МОДИФИЦИРОВАННЫХ ФЛЮОРЕСЦЕИНОВ ДЛЯ ИСПОЛЬЗОВАНИЯ В КЛИК-РЕАКЦИЯХ.

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Розработано методологию синтеза модифицированных флуоресценов для использования в клик-реакциях. Были улучшены выходы описанных стадий синтеза и оптимизированы способы выделения и очистки конечных и промежуточных соединений.

Ключевые слова: флуоресцений, азидзамещенный флуоресцен, клик реакция.

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